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OPIOID RECEPTOR IN VASCULAR SMOOTH MUSCLE

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of philosophy, The City University of New York.

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Abstract

OPIOID RECEPTOR IN VASCULAR SMOOTH MUSCLE

By

Rubina W. Saeed

Advisor: Professor Harold I. Magazine

Opioid receptors have been extensively studied in the nervous system of mammals and invertebrates. Recently they have been demonstrated in inflammatory cells and in gastric smooth muscle cells. Opiates and endogenous opioids have been demonstrated to modulate blood pressure and responses to hypotension and hypothermia. Although these data suggest the potential for direct opioid regulation of vascular tone, opioid receptors have not been demonstrated in vascular smooth muscle. We used RT-PCR to evaluate delta opioid receptor (DOR) expression in the rat arterial smooth muscle cell line, A-10. We amplified and isolated a DNA fragment of expected size. Cloning and sequencing of the fragment revealed a sequence identical to the known DOR sequence from rat brain. Pharmacological studies were performed using human and rat vascular tissue. DOR competition studies demonstrated the presence of delta opioid selective receptors in vascular smooth muscle that was consistent with the δ_2 subtype. Immunocytochemical analysis using commercially available DOR antisera demonstrated the presence of DOR-binding site on A-10 cells.

To investigate receptor-signaling mechanisms, we employed confocal microscopic analysis and evaluated changes in membrane potential and intracellular calcium in response to DOR-activation. The DOR-selective agonist, DAMA, induced a 11.5mV membrane depolarization that was potentiated and inhibited by preincubation with the tyrosine phosphatase inhibitor, vanadate, and the tyrosine kinase inhibitor, erbstatin, respectively. Exposure of A-10 cells to DAMA stimulated an increase in intracellular calcium that was linked to ryanodyne-sensitive stores and extracellular calcium. Physiological characterization of the DOR was performed by evaluation of rat aortic rings. DAMA stimulates the contraction of rat aorta whereas preincubation with ryanodyne and naltrindole, potentiated and inhibited this response respectively.

These data taken together, demonstrate the presence of a functional DOR in vascular smooth muscle that is couple to the direct regulation of contractile tone.

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INTRODUCTION

I-HISTORICAL PERSPECTIVE

The analgesic and antidiarrhoeal effects of opium were known to predynastic Egyptians and Sumerians. In early 19th century, after 5000 years of its medicinal use, opium was replaced by morphine, a highly active compound in opium. Since the discovery of its high potential for abuse, several attempts have been made to isolate a safer and less addictive analogue of morphine. To date, however, no claims have been made for an abuse-free compound. Nevertheless, the search for a safer compound led to the synthesis of a number of opioid peptides and the discovery of endogenous opioids and their receptors (Jordan B, *et al.*, 1998). The concept of pharmacologically relevant receptors for opioids started in the early 1950s, when Beckett and Casey first elaborated the activities of opioid stereoisomers. Several years later in 1965 Portoghese PS, *et al.*, introduced the concept of several modes of interactions of morphine and other endogenous compounds with opioid receptors. Subsequently, Goldstein *A, et al.*, in 1971 proposed the concept of using radiolabelled analgesic compounds to demonstrate the existence of opioid receptors. Following the introduction of highly specific opioid radioligands, three independent groups simultaneously demonstrated the presence of stereospecific opioid binding sites in mammalian brain (Pert CB, *et al.*, 1973; Simon EJ, *et al.*, 1973; Terenius L, *et al.*, 1973). Since then, significant efforts have been made towards the understanding of opioid

receptors and their biochemical and pharmacological effects. Several investigators have isolated and purified opioid receptor proteins confirming their existence on the cell surface. Work presented in this dissertation contributes to our knowledge regarding δ-opioid receptor function and demonstrates for the first time the presence of DOR in arterial smooth muscle (We used rat and human arterial tissue and rat aortic smooth muscle cell line and therefore demonstrating the presence of delta opioid receptor (DOR) in arterial smooth muscle).

II-DISCOVERY OF OPIOID RECEPTOR

Martin WR, *et al.,* 1976 has postulated three different types of opioid receptors. The convincing evidence came from their behavioral and neurophysiological observations in dog. The newly discovered receptors were named after the drug used in the study i.e., μ, for morphine; κ, for ketocyclazocine and σ, for SKF 10,047 or N-allylnormetazocine. Soon after the availability of radiolabelled enkephalins (enkephalins are the peptides that simulate the pharmacological effect of morphine and contain the sequence, Tyr-Gly-Gly-Phe-Met), Kosterlitz HW and coworkers (1975) looked for the binding sites of these compounds in peripheral tissues and two years later Lord JAH, *et al.,* (1977) discovered the fourth type of opioid receptor in the mouse vas deferens and named it δ, for deferens. Because of the fact that σ-receptor subsequently turned out to be non-opioid in nature, there are only three major types of opioid receptors μ, δ and κ. All three of these have been cloned and their binding and functional characteristics are consistent with their identities as μ, δ and κ receptors (Reisine T, *et al.,* 1993; Kieffer BL, *et al.,* 1995 and Satoh M, *et al.,* 1995). These receptors have been reclassified by an International Union of Pharmacology subcommittee and are now known as OP1 (δ), OP2 (κ) and OP3 (μ).

Furthermore, a number of reports support the existence of additional receptor types, particularly the epsilon receptor (ε, Wuster M, *et al.,* 1979) the lambda receptor (λ, Grevel J, *et al.,* 1985) and the zeta receptor (ζ, Zagon IS,

et al., 1991). Among these, ε-receptor has been studied in greater detail in rat vas deferens. However, molecular evidence is needed to establish a convincing identification.

III-OPIOID RECEPTOR SUBTYPES

Pharmacological studies using a variety of opioid receptor agonists and antagonists revealed the presence of receptor subtypes. So far, three subtypes for μ-receptor: μ_1, μ_2 (Pasternak G, *et al.*, 1986), and μ_3 (Stefano GB, *et al.*, 1995); two subtypes for δ-receptor: δ_1, δ_2 (Portoghese PS, *et al.*, 1992; Zaki PA, *et al.*, 1996); and three subtypes for κ-receptor: κ_1, κ_2 (Zukin RS, *et al.*, 1988), κ_3 (Clark JA, *et al.*, 1989) are known (See table–1). These subtypes are not characterized at the molecular level, yet one report (Rossi GC, *et al.*, 1997) supported the existence of δ subtypes using an antisense approach to map the DOR-1 sequence in mice.

Table-1: **Based upon the radioligand binding studies, following table shows the type of opioid receptor and their ligands.**

Opioid receptor	Preferential endogenous opioid ligands	subtypes	Preferential ligands	References
δ	Enkephalins	δ1	DPDPE[1]	Vanderah *et al.*, 1994
		δ2	DSLET[2] & DAMA[3]	Portoghese *et al.*, 1992
κ	Dynorphins	κ_1	U-69,593[4]	Lahti *et al.*, 1985
		κ_2	N/A	
		κ_3	N/A	Clark *et al.*, 1989
μ	β-endorphin	μ_1	DAMGO[5]	Pasternak *et al.*, 1986
		μ_2	TRIMU-5[6]	Tive *et al.*, 1992
		μ_3	Morphine[7]	Stefano *et al.*, 1993

[1] Tyr-D-Pen-Gly-Phe-D-Pen
[2] Tyr-D-Ser-Gly-Phe-Leu-Thr
[3] Tyr-D-Ala-Gly-Phe-Met-NH_2
[4] (5α,7α,8β)-(-)-N-methyl-N[7-(1-pyrrolidinyl)-1-oxspiro(4,5)dec-8-yl] phenyl-benzenacetamide
[5] Tyr-D-Ala-Gly-MePhe-Gly-ol
[6] Tyr-D-Ala-Gly-NH-$(CH_2)_2$-$CH(CH_3)_2$
[7] (5α,6α)-7,8-didehydro-4,5-epoxy-17-methylmorphine-3-6-diol

IV-STRUCTURAL CHARACTERISTICS OF OPIOID RECEPTORS

Hydrophobicity analysis of the deduced protein sequence of the cloned opioid receptors has indicated the presence of putative seven transmembrane domains, characteristic of G-protein coupled receptors (Probst WC, *et al.*, 1992). A membrane topographical model of rat mu-opioid receptor (MOR) is shown in Fig-1 (Satoh M, *et al.*, and Kieffer BL, *et al.*, 1995). It is important to note that these receptors are highly homologous. Overall the amino acid sequence of all three members of opioid receptor family are ~ 60% identical to one another. The amino acid sequence identities of all three receptors are given in table-2 (Satoh M, *et al.*, 1995). Higher identities are found in the transmembrane (73-76%) and in intracellular regions (63-65%) while lower identities (34-40%) are located in the extracellular region.

Further analysis of the receptor's primary structure confirms that opioid receptors are classical members of seven transmembrane G-protein gene family as they have been demonstrated to contain the conserved proline and aromatic amino acid residues (Fig-1). They also contain potential glycosylation sites at the N-terminus and a consensus sequence for phosphorylation in the third intracellular loop and C-terminal domain. Both glycosylation and phosphorylation sites are classically described post-translational signals. Removal of the N-terminus does not affect the ligand binding or signal transduction (Wang JB, *et al.*, 1993; Kong H, *et al.*, 1994) but slightly decreases the expression level (Surrat CK, *et al.*, 1994). The putative

glycosylation site might play a role in protein targeting at the cell surface without affecting the receptor function, as reported for muscarinic (Liles WC, *et al.*, 1986) and β-adrenergic receptors (Dixon RA, *et al.*, 1987). In addition, putative phosphorylation sites are important for the regulation of receptor activity by intracellular kinases (Lefkowitz RJ, *et al.*, 1990).

Table-2. Amino Acid identities among the three subtypes of rat opioid receptors.

	Amino acid identity		
	Chimeric receptors		
	μ/δ %	μ/κ %	δ/κ %
Extracellular regions	34	36	40
N-terminal	25	33	31
First loop	72	67	72
Second loop	42	35	54
Third loop	18	18	11
Transmembrane (TM) regions	76	73	74
TM1	69	62	62
TM2	100	84	84
TM3	82	91	91
TM4	45	32	55
TM5	79	79	75
TM6	77	73	68
TM7	86	95	90
Intracellular regions	63	66	63
First loop	90	100	90
Second loop	91	91	82
Third loop	87	87	83
C-terminal (before palmitoylation)	82	91	82
C-terminal (after palmitoylation)	27	31	21
Total	58	59	61

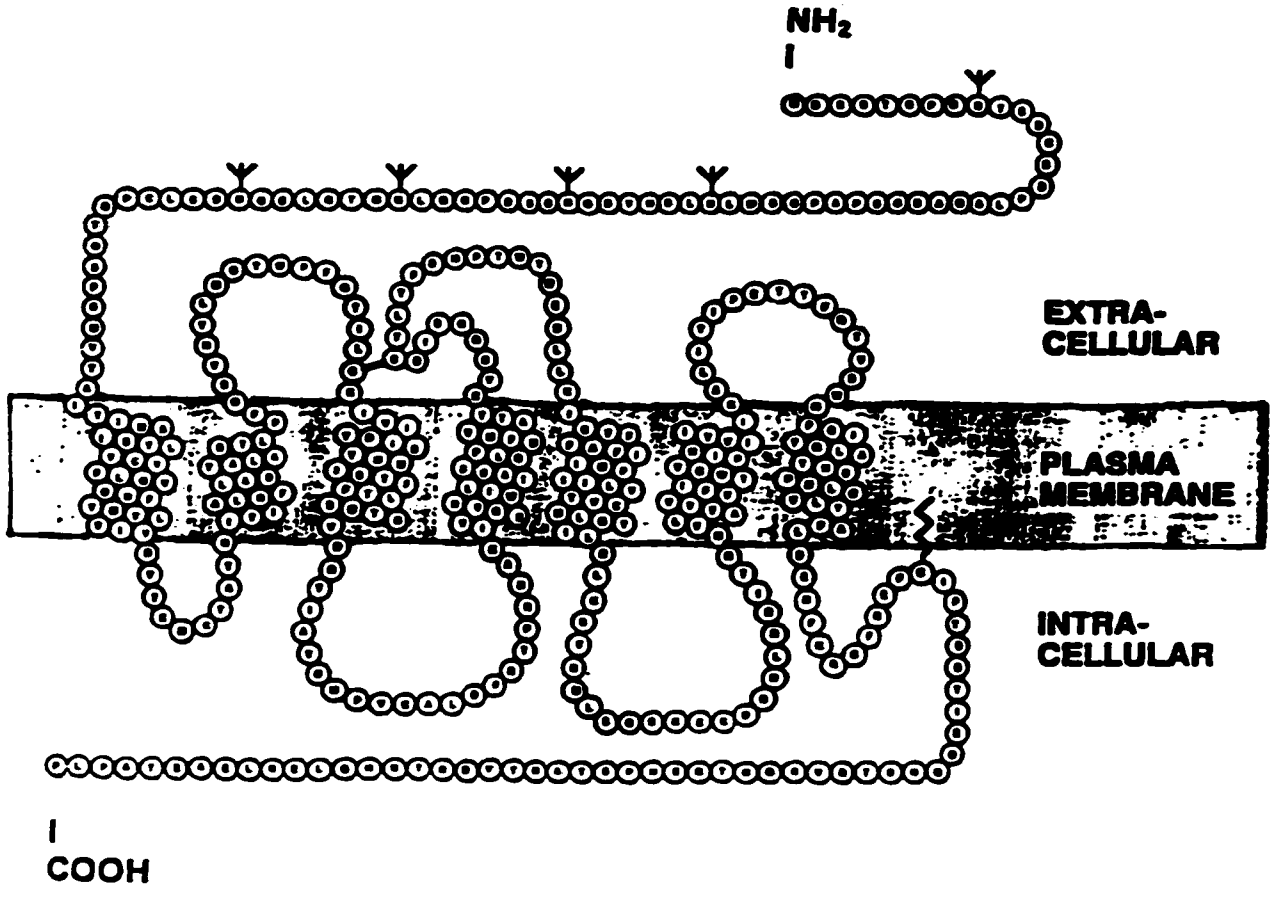

Fig-1: Topographical model of the rat mu-opioid receptor (Satoh M *et al.*, 1995). Potential sites for N-linked glycosylation and palmitoylation are shown as branched and zigzag structures respectively. Cysteine residues in the first and second extracellular loops are considered to be involved in disulphide linkages.

V-OPIOID PEPTIDES

Opioid peptides are a family of endogenous structurally related compounds. They work as neuromodulators and play a major role in noniceptive pathways. The first two members of the family were isolated from brain where they are most abundant (Hughes J, *et al.*, 1975). Since then more than 22 peptides have been identified from brain, adrenals and pituitary glands. These peptides are derived from large precursors which are translated from three different genes and are named preproopioimelanocortin (POMC), preproenkephalin and prodynorphin (Rossier J, *et al.*, 1993; Young E, *et al.*, 1993; Day R, *et al.*, 1993). These peptides contain a common N-terminal sequence (NH_2-Tyr-Gly-Gly-Phe-Met/Leu).

VI-OPIOID RECEPTOR BINDING

The binding of a novel ligand to its specific receptor is the first step required to initiate a particular response. Structure function analysis of the cloned opioid receptor has been very useful in identifying amino acid residues and domains of the receptor involved in ligand binding. Chimeric protein analysis has been employed to assess the structural requirements at the receptor-binding site. Furthermore, Meng F, *et al.*, (1995) have shown that replacement of κ sequences up to transmembrane-3 (tm3) in the delta opioid receptor has minimal effects on delta ligand binding, suggesting very little involvement of the first 3 transmembrane regions in delta selectivity.

Kong H and coworkers (1994) demonstrated that the N-terminal region of KOR is crucial for an antagonist binding. A chimeric protein in which the N-terminal region of DOR is replaced by N-terminal of KOR has high affinity for κ antagonist, suggested that the N-terminal of KOR contains antagonist binding domains. On the other hand, delta agonist and antagonist did not show any change in binding affinities, demonstrating that DOR N-terminal is not required for δ ligand binding. Similar results have been obtained for μ receptor by Surrat CK, *et al.*, (1994), in which they demonstrated that κ antagonists bound to its receptor in a distinct manner than other opioid receptors. Xue JC, *et al.*, (1994) and Wang JB, *et al.*, (1994c) demonstrated that the 2nd extracellular loop of κ opioid receptor is crucial for specific agonist binding. A chimeric μ/κ receptor in which this loop is replaced by second extracellular loop of μ

receptor binds with dynorphin-A suggest that a relatively restricted region of κ receptor is needed for selective agonist binding. Collectively, these data have shown that tm3 of DOR (Varga EV, *et al.*, 1996), tm1 and tm3 of MOR (Wang WW, *et al.*, 1995 and Minami M, *et al.*, 1995) and tm2, tm4 of KOR (Kong H, *et al.*, 1994; Meng F, *et al.*, 1995; Wang JB, *et al.*, 1995; Xue JC, *et al.*, 1994), are important in their respective agonist selective binding.

In order to determine the crucial amino acid residues necessary for binding, mutant receptors with single amino acid substitution were used. Kong H, *et al.*, 1993 demonstrated that a conserved aspartate in transmembrane 2 of delta opioid receptor was critical for Na^+ regulation and for high affinity agonist binding. Additionally, antagonist binding to a mutant in which this aspartate was replaced by asparagine did not affect the binding compared to the wild type, while most agonist had low affinity for the mutant receptor. Similar results were obtained when a conserved aspartate in the tm3 of the delta receptor was converted into an asparagine, suggesting that the negative charges provided by these two aspartates may play a role to serve as counterions to cationic groups of delta agonists to stabilize binding through electrostatic interactions. Bot G, *et al.*, 1998 demonstrated the importance of a single amino acid in binding, as they have shown that mutagenesis in Asp114 in the rat mu-opioid receptor differentiates ligand binding. Claude PA, *et al.*, (1996) revealed that a single serine residue in all three receptors (Ser196 at μ, Ser177 at δ, Ser187 at κ) carries full agonist properties on classical antagonist

such as naloxone. Collectively aromatic transmembrane resiudes at positions 129 and 308 (Befort K, *et al.*, 1996), and at positions 95, 284, 296 and 297 (Kong H, *et al.*, 1993; Valiquette M, *et al.*, 1996; Spivak CE, *et al.*, 1997), of the δ opioid receptor are necessary for selective ligand binding.

Thus one can easily imagine that a small change in a receptor structure, modifies its binding to the ligand and ultimately its affect. This is a major obstacle in obtaining only single receptor subtype activation in *vivo*. Therefore, appropriate caution should be taken to draw a conclusion for a receptor subtype.

VII-MECHANISM OF OPIOID RECEPTOR SIGNAL TRANSDUCTION

The mechanism of opioid receptor signal transduction begins with the receptor activation followed by stimulation of second messenger molecules. Fortunately, the cloning of opioid receptors has provided us a roadway to study these pathways. So far, we know that opioids transduce their signals by G-protein-coupled receptor.

Opioids are bimodal in effect *i.e*, they cause inhibitory as well as excitatory effects. Binding of opioid receptors with a variety of G-proteins demonstrates the complexity of the system. They can bind to G_s, G_o, G_i and G_q proteins. To date, several isoforms of each subunit have been identified. To further complicate the situation μ and δ opioid receptors may couple to the same effector system *via* different G proteins (Neer E, *et al.*, 1995; Connor M, *et al.*, 1999; Law PY, *et al.*, 1999). They also modulate a variety of intracellular molecules such as cAMP, IP_3, Ca^{2+} and K^+ channels.

VIII-BIMODAL EFFECTS OF OPIOIDS

Biological effects of opioid binding to its receptor leads to a variety of responses. These include analgesia, miosis, sedation, hypothermia, bradycardia, insensitivity, and depression. The role of opioids in pain control has been extensively studied for the last few decades and so far its ability to inhibit neurotransmitter release is the only known explanation for its effects (MacDonald RL, *et al.*, 1978 and Mudge A, *et al.*, 1979). This inhibitory effect is achieved by inhibition of voltage-gated calcium channels, activation of K^+ currents and reduction in cAMP levels. All these effects are known to cause an inhibition in neurotransmitter release. Recently, opioids have also been demonstrated to cause stimulation of neurotransmitter release (Sarne Y, *et al.*, 1996). This enhancement has been achieved by stimulatory actions of a variety of second messenger molecules, including elevation of intracellular Ca^{2+}, increase in adenylate cyclase activity and IP_3 hydrolysis (Cahill CM, *et al.*, 1993; Craine SM, *et al.*, 1990, 1992, 1996; Lin Y, *et al.*, 1994). The bimodal effects of mu and delta opioids agonist on enkephalin release have recently been demonstrated in the pallidum of the freely moving rat (Olive MF, *et al.*, 1998). Additionally, endogenous peptides are also known to modulate endocrine processes and the immune system (Maggi R, *et al.*, 1995; Genazzani AR, *et al.*, 1981; Brown S, *et al.*, 1974 and Roy S, *et al.*, 1996). The question arises as to how these peptides are able to effect so many systems. The cloning of all three receptors should be a remarkable tool to

study the pathways initiated by discreet receptor activation. The use of cloned opioid receptors and the regulation of effector systems in conjunction with inhibitory and stimulatory effects will be discussed in the upcoming session.

A-Inhibitory Effects of Opioids

1- Inhibition of Adenylate Cyclase Activity: Blume A, *et al.,* in 1979 first reported the association of guanine nucleotides with opioid receptors in brain cell membranes and in neuroblastoma X glioma (NG108-15) hybrid cells. Opioids exist in two different states depending upon the presence or absence of guanine nucleotides as shown by Childers S, *et al.,* in 1980. He showed that although the addition of GTP caused an increase in both agonist association and dissociation, the increase in dissociation is much greater. Sharma S, *et al.,* in 1975 demonstrated a GTP-dependent inhibition of adenylate cyclase by morphine in a naloxone reversible manner. Blum A, *et al.,* in 1978 revealed that opioid stimulated GTPase activity in NG108-15 cells, further providing evidence of opioid receptor coupling with G-protein. Later, In 1984 Franklin P, *et al.,* revealed the regional differences between the distribution of GTPase activity and adenylate cyclase inhibition. The GTPase activity induced by opioids is pronounced in cortex, hippocampus and striatum while inhibition of adenylate cyclase appears to occur in the striatum only.

Stimulation of cloned rat, mouse and human κ receptors expressed in COS or PC12 cells lead to the inhibition of cAMP formation (Wang JB, *et al.,*

1994b; Meng F, *et al.*, 1993; Kong H, *et al.*, 1994; Yasuda K, *et al.*, 1993; Tallent M, *et al.*, 1994; Xie GX, *et al.*, 1994). Similarly, cloned rat and human μ-opioid receptor in COS and CHO cell lines significantly reduced both the forskolin stimulated adenylate cyclase and the production of inositol triphosphate, in a naloxone reversible manner (Chen Y, *et al.*, 1993a; Wang JB, *et al.*, 1994b; Raynor K, *et al.*, 1995). A similar observation was achieved with receptors stimulated by delta agonist from cells exposed to forskolin (Evans CJ, *et al.*, 1992; Kong H, *et al.*, 1993; Yasuda K, *et al.*, 1993).

The inhibition of adenylate cyclase is consistent with information obtained from tumor cell lines. In NG108-15 hyridoma cell line, the activation of delta receptor inhibits adenylate cyclase activity by $G_{\alpha i2}$ protein (McKenzie FR, *et al.*, 1990). Additionally, two other G-proteins also known to interact in delta specific activation for adenylate cyclase inhibition *i,e.* $G_{\alpha 02}$ and $G_{\alpha i3}$ (Roerig SC, *et al.*, 1992 and Prather PL, *et al.*, 1994). In *Xenopus oocytes*, β_2-adrenoceptor mRNA was co-injected with mouse delta receptor mRNA. Upon activation, delta agonist caused an inhibition of the isoprenaline-induced increase of cAMP production in a naltrexone-reversible manner (Tamir M, *et al.*, 1993). In 1997 Clark MJ, *et al.*, demonstrated that C6 glioma cell lines stably expressed homogenous population of DOR and the activation of this receptor strongly inhibits the forskolin stimulated adenylate cyclase by G_o/G_i proteins. This effect is successfully blocked by pertussis toxin, again suggesting mediation *via* G_o/G_i proteins (Hsia J, *et al.*, 1984).

Inhibition of adenylate cyclase activity by delta and mu opioid receptor agonists has also been demonstrated in brain tissue (Chneiweiss H, *et al.*, 1988 and Polastron J, *et al.*, 1990). Dual function of δ and μ but not κ-receptor agonists have been observed in rat olfactory bulb, in which opioids increased basal adenylate cyclase activity while inhibiting the enhanced cAMP production possibly by differential actions on the various forms of enzyme (Onali P, *et al.*, 1991; Olianas MC, *et al.*, 1992, 1994). In contrast coupling of delta receptor to adenylate cyclase caused an inhibition of phospholipase C activity in guinea pig brain membranes (Misawa H, *et al.*, 1990, 1995).

2- *Reduction in Intracellular Ca^{2+} Level:* The calcium ion is another second messenger molecule that regulates a variety of cellular responses including neurotransmission. Calcium can enter into the cell from extracellular sources by voltage-gated channels after depolarization or it can be released from intracellular stores after agonist stimulation. All three opioid receptors are known to cause an inhibition of voltage-gated Ca^{2+} channels (Childers SR, *et al.*, 1991; North A, *et al.*, 1991; Nestler EJ, 1992; Huang LM, *et al.*, 1995). This effect is blocked by PTX, suggesting the involvement of G_o/G_i proteins (Seward E, *et al.*, 1991; Surprenant A, *et al.*, 1990; Law PY, *et al.*, 1995). The G proteins involved in this inhibition are cell-type dependent. In smooth muscle cells, G_o/G_i proteins are primarily involved (Murthy KS, *et al.*, 1995) while in cardiac myocytes this effect is mediated by G_q proteins (Tai KK, *et al.*, 1992).

So far this effect has been found in a variety of cell lines and neuronal preparations (Seward E, *et al.*, 1991; Gross RA, *et al.*, 1987; Surprenant A, *et al., 1990;* Huang LM, *et al.*, 1995; Rusin KI, *et al.*, 1997). A number of calcium channel subtypes have been reported to be involved in opioid-induced calcium inhibition: N and P/Q-type Ca^{2+} channels (Rhim H, *et al.*, 1994; Tallent M, *et al.*, 1994), T-type Ca^{2+} channels (Rusia K, *et al.*, 1995), and L-type Ca^{2+} channels (Piros E, *et al.*, 1995). L-type coupling has not yet been found in brain cells (Tallent M, *et al.*, 1994). It is perplexing that a variety of channels are involved to induce the same effect and what makes a cell prefer one channel type over the other is not yet known. Cloning of calcium channel subtypes and opioid receptors made it possible to look for a possible explanation in a heterologous expression systems. A number of factors appear to be involved. First, L-type is far less frequently expressed and thus its coupling can easily be overshadowed by higher coupling levels of N and P/Q type. Second, the transduction of L-type channels may require a specific G-protein coupling, not present in certain cell types. Third, alternative splicing of one subunit can give rise to a number of isoforms of the same channel-type and thus cause differential susceptibility to modulation by opioid receptors (Piros E, *et al.*, 1996; Bourinet E, *et al.*, 1996). These studies suggest that choice between various calcium channel subtypes may depend upon the cellular environment.

3- Activation of K^+ channels: The third known cellular event that contributes to the inhibition of neurotransmitter release is the activation of inwardly rectifying K^+ channels. This response leads to membrane hyper-polarization and an increase in K^+ conductance (North A, 1986; Law PY, *et al.*, 1995). This activity has been reported in a variety of preparations such as hippocampus (Wimpey TL, *et al.*, 1991), gelatinosa neurons (Grudt TJ, *et al.*, 1993) submucosal plexus and locus coeruleus (North RA, *et al.*, 1987). This effect is also PTX-sensitive suggesting the involvement of G_o/G_i proteins (Tatsumi H, *et al.*, 1990). In addition, all three opioid receptors have been demonstrated to cause activation of inwardly rectifying K^+ channels in heterologous systems, primarily in *xenopus oocytes* and this increase in K^+ conductance is also blocked by PTX (Dascal N, *et al.*, 1993; Chen Y, *et al.*, 1994b; Henry D, *et al.*, 1995).

B-Stimulatory Effects of Opioids

The first evidence that showed the excitatory effect of opioids was reported in 1977 by Vizi ES, *et al.*, They demonstrated that this effect is due to the blockage of inhibitory mechanism of opioids and thus called the "disinhibition" mechanism (Hirst RA, *et al.*, 1998). For example, in the caudate nucleus, the stimulatory effect of opioids on acetylecholine is due to the presynaptic suppression of inhibitory dopaminergic system. But recently several scientists have been able to demonstrate a more direct stimulatory

effect of opioids in neurons (Crain SM, *et al.*, 1990; Lin Y, *et al.*, 1994; Shen KF, *et al.*, 1994). The physiological significance of the stimulatory effects of opioids is not known yet and presently a subject of extensive debate. The role of opioids in excitatory effects and the involvement of second messenger molecules will be discussed next.

1-Increase in Adenylate Cyclase Activity: The stimulatory effect of opioids on adenylate cyclase was first reported in mid 1970s (Collier HOJ, *et al.*, 1975; Sharma S, *et al.*, 1975; Traber J, *et al.*, 1975). This effect has been demonstrated in a number of preparations including heart and neuronal tissue (Lee AYS, *et al.*, 1987 and Mackman MH, *et al.*, 1988). Later Federman AD, *et al.*, 1992 reported the stimulation of adenylate cyclase through the $\beta\gamma$ subunit of $G_{i(o)}$-protein. A few years later, involvement of the $\beta\gamma$ subunit of type-II adenylate cyclase was reported (Kaneko S, *et al.*, 1994b; Olianas MC, *et al.*, 1993 and 1994). Concurrently some studies demonstrating the direct stimulatory effect of opioids involving G_S-protein in the activation of adenylate cyclase (Crain SM, *et al.*, 1990; Shen KF, *et al.*, 1994). The proposed mechanism for opioid activity is that the adenylate cyclase leads to the accumulation of cAMP, which in turn activates protein kinase A. PKA will produce an increased level of phosphorylation altering the physiological activity of the cell. Activation of transfected kappa opioid receptor into *xenopus oocytes* caused an increase in cAMP production (Kaneko S, *et al.*, 1994). This

stimulation results from the activation of type-II adenylate cyclase *via* the $G_{\beta\gamma}$-proteins (Tsu RC, *et al.*, and Chan JSC, *et al.*, 1995). Similarly mu-opioid receptor activation stimulates cAMP formation in a calcium/calmodulin (Ca^{2+}/CaM) dependent pathway at low doses (nM concentrations) while at high doses (0.1μM concentrations) it causes an inhibition of adenylate cyclase. This bimodal phenomenon has also been found in the myenteric plexus (Wang L, *et al.*, 1995). This is an important finding and may have a great impact on research directed towards the opioid related physiological response. Recently, it has been demonstrated that the opioid action of adenylate cyclase is isozyme specific. For example, the mu opioid receptor stimulates type II, IV and VII adenylate cyclase while it inhibits type I, V, VI and VIII (Avidor-Reiss T, *et al.*, 1997).

2- *Increase in Phosphoinositide Hydrolysis:* Endogenous peptides as well as cloned opioid receptors have been demonstrated to stimulate IP_3 production (Smart D, *et al.*, 1995 and 1996; Jin W, *et al.*, 1994; Sanchez-Blazquez P, *et al.*, 1998; Sanchez-Blazquez P, *et al.*, 1999). This activity has also been shown in heterologous systems (Johnson PS, *et al.*, 1994). It has been reported that opioids mobilize calcium from inositol 1,4,5-triphosphate-sensitive stores in NG108-15 cells (Jin W, *et al.*, 1994). Furthermore, this Ca^{2+} mobilization from internal stores is mediated by pertussis toxin (PTX) sensitive G-proteins, suggesting a role for the $G_{I(o)}$-protein (Okajima F, *et al.*, 1993; Tsu

RC, *et al.*, 1995). Additionally the stimulation of mu receptor in SH-SY5Y cells activates PLC while delta receptor caused liberation of $\beta\gamma$ subunit of G_i and failed to induce PLC. This delta receptor activity did not effect the mu-induced $Ins(1,4,5)P_3$ formation (Smart D, *et al.*, 1995) suggesting that $\beta\gamma$ subunit of G_i is not involved in PLC activation in this cell type. It may be concluded that two different PLC isoforms are involved as activators of PIP_2 hydrolysis.

3-Increase in Intracellular Calcium levels: Opioid-induced intracellular calcium increase may come from extracellular sources or from intracellular stores or both (Tang K, *et al.*, 1994; Jin W, *et al.*, 1994; Tai KK, *et al.*, 1992; Steine-Martin A, *et al.*, 1993; Smart D, *et al.*, 1994, 1995 and 1996; Wandless A, *et al.*, 1996; Furuichi *et al.*, 1995). The first few studies about intracellular calcium increase were reported in the early 80s. Higashi H, *et al.*, 1982 reported the increase in intracellular calcium in response to morphine. A few years later Lorentz M, *et al.*, 1988 reported the morphine induced activation of calcium channels. Craine SM, *et al.*, 1996 demonstrated that opioids increase the calcium spike of the action potential in the dorsal root ganglion neurons and this response is G_S dependent. Smart D, *et al.*, in 1995 demonstrated that mu opioid receptor activation stimulates phospholipase activity *via* calcium channel opening in SH-SY5Y cell lines as does delta receptor in CHO cell line (Harrison C, *et al.*, 1997). A similar observation was achieved in a heterologous system of *Xenopus oocyte* when kappa opioid receptor was

activated (Kaneko S, *et al* 1994). Two years later (1996) Smart D and his associates demonstrated that delta opioid receptor in NG108-15 cells stimulate IP_3 formation and mobilizes calcium from intracellular stores. Taken together these studies suggest the involvement of two pathways of intracellular calcium increase, a PLC mediated pathway and an IP_3 mediated pathway. Recently, it has been suggested that there is a connection between IP_3 /Ca^{2+} and cAMP signaling as Zhang and his co- workers reported that kappa opioid receptor activation caused cAMP inhibition by phosphoinositol/Ca^{2+} pathway (Zhang WM, *et al.*, 1998).

The phenomenon of synergism is well documented in the literature as a number of researchers reported the elevation of opioid-induced increase in intracellular calcium in the presence of other receptors such as bradykinin or P2 purinergic receptors (Okajima F, *et al.*, 1993), and ryanodyne receptors (Allouche S, *et al.*, 1996).

Several types of calcium channels may be involved in intracellular calcium increase. A number of studies have shown the involvement of L-type Ca^{2+}channels (Eriksson PS, *et al.*, 1993; Smart D, *et al.*, 1995; Tang K, *et al.*, 1994). In addition, Karen O and his co-workers (1997) demonstrated the involvement of N-type Ca^{2+} channels. Further work is needed to determine if other channel types are also involved.

IX-OPIOID RECEPTOR IN SMOOTH MUSCLE

First direct evidence of opioid receptors of smooth muscle was reported almost a decade after its activity was shown in intestinal smooth muscle (Kachur JF, *et al.*, 1980; Burks TF, *et al.*, 1983), and in gastric smooth muscle (Ruckebusch Y, *et al.*, 1984). In early 1990s, two laboratories identified the receptors in intestinal and gastric smooth muscle (Grider JR, *et al.*, 1991; Zhang L, *et al.*, 1992). Since then a number of publications have shown their activity, localization and effect in these tissue types (Bagnol D, *et al.*, 1997; Champion HC, *et al.*, 1998; Louise CN, *et al.*, 1996). Additionally, delta opioid receptor transcript has recently been demonstrated in rat heart (Wittert G, *et al.*, 1996). The identification of these receptors in smooth muscle in combination with rapidly emerging data supporting opioid-induced cardiovascular activities (see conclusion), suggested that these receptors may be present in vascular smooth muscle.

In the present thesis, we focused on delta receptor due to its well-established role in tissue hypoxia (Cornfield DN, *et al.*, 1994).

CHAPTER ONE:

MOLECULAR CHARACTERIZATION OF DOR

INTRODUCTION

MATERIAL AND METHOD

RESULTS

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Chapter One:

Molecular Characterization of DOR

INTRODUCTION

Molecular cloning of the delta opioid receptor has been reported in mouse, rat and human. In the mouse, it has been cloned in neuroblastoma X glioma (NG108-15) hybrid cells (Evans CJ, *et al.*, & Kieffer BL, *et al.*, in 1992), in thymocytes (Sedqi M, *et al.*, 1996) and in brain (Yasuda K, *et al.*, 1993). In the rat, the DOR has been cloned in cerebrum (Fakuda K, *et al.*, 1993; Abood ME, *et al.*, in 1994). In human, it has been cloned in striatum/cortex (Knapp RJ, *et al.*, 1994) and in SH SY5Y cells (Simonin F, *et al.*, 1994) and recently, it has also been cloned in zebrafish brain (Barrallo A, *et al.*, 1998).

So far, no reports have been made for cloning a DOR in vascular smooth muscle: our data is the first demonstration in this regard. The detection and sequencing of a receptor can be accomplished using reverse transcription polymerase-chain reaction followed by DNA purification and sequencing. We also employed RT-PCR followed by cloning of the amplified fragment prior sequencing to commercial sources (Rockefeller University's DNA sequencing facility, New York, NY).

We decide to perform this assay with cell line (using rat aortic smooth muscle cell line, A-10), for two reasons. 1) a cell line contains a homogenous population of a single cell type so that there is no chance of cell contamination

from other tissue, 2) RT-PCR is a powerful technique and even a protein of low expression can be identified.

MATERIAL AND METHOD

RT-PCR, Cloning & Sequencing: RT-PCR was performed on rat aortic smooth muscle cells (American Type Culture Center., MD). The clonal cell line, A-10, was derived from thoracic aorta of embryonic rat and cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., MO), containing 20% fetal bovine serum (FBS, Gibco BRL., MD). Cells were fed with fresh medium every 3-4 days. This is an adherent cell line and cells were trypsinized and washed (Biofluids., MD), prior re-culturing. The cell line was cultured only for 12 passages.

Three million cells were harvested and lysed in Ultraspec^{TM} RNA reagent (Biotecx Lab. Inc., Texas), to isolate the total cytoplasmic RNA. Isolated RNA was reverse transcribed into cDNA by using 0.5μg/μl oligo(dT), 200units/μl superscript reverse-transcriptase and 2units/μl RNase-H. The resulting cDNA was then used to perform PCR (35 cycles, 96^{O}C/min, 60^{O}C/min, 72^{O}C/1.5 min), on a deltacycler II system (Ericomp Inc., CA). Oligonucleotide primers based on bp 438-462, forward primer (5′-CAACATGTTCACCAGCATCTTCACG-3′) and bp 1035-1012, reverse primer (5′-GAAGCAGCGCTTGAAGTTCTCGTC-3′) of rat DOR-1 (Abood ME, *et al.*, 1994), were used in the PCR. The PCR-product was then electrophoresed on 1% agarose gel. This band was then gel purified (Qiagen Products, CA) and subcloned by using the pGEM-T Easy Cloning Vector. The clone was then amplified in liquid culture for plasmid purification. The insert was released by

Eco RI digestion (Promega Corp., WI) and purified by agarose gel electrophoresis. This cDNA clone was sequenced (Rockefeller Univ. DNA sequencing facility, New York, NY) and subjected to GeneBank analysis to determine % homology.

RESULTS

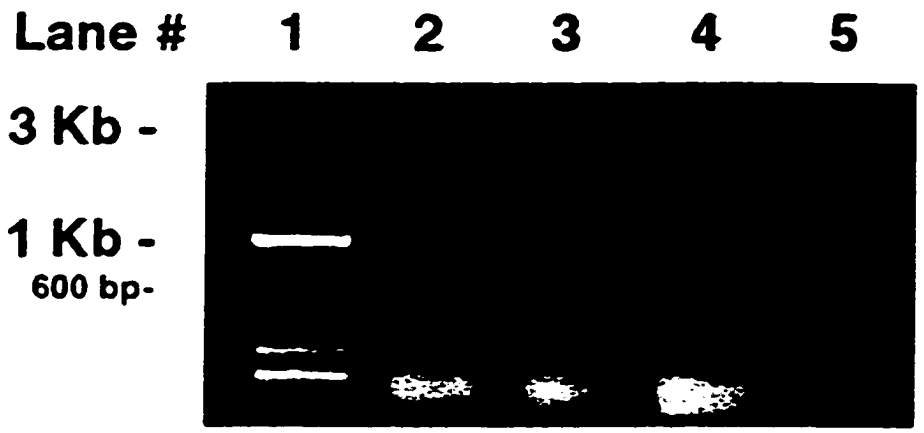
The integrity of the isolated RNA was confirmed by agarose gel electrophoresis, followed by ethidium bromide staining prior to performing RT-PCR. The amplification produced a DNA fragment of the expected size (597 bp) in the presence of forward and reverse primers only (lane-4), (See Fig–2). In contrast no product was amplified in the absence of primer A or B or in the absence of enzyme reverse transcriptase (lane 2, 3 and 5 respectively), confirming, that the band is indeed an RT-PCR product and not merely a contamination of genomic DNA. Our sequencing data demonstrates that the cloned DNA fragment is identical to the known rat DOR sequence demonstrating the presence of a DOR-1 transcript in rat vascular smooth muscle.

We evaluated the homology of the cloned fragment to mu and kappa opioid receptor sequences (Table–3). Comparison data revealed 33.7% homology with MOR and 21.4% homology with KOR, consistent with the cloning of a DOR DNA fragment.

DISCUSSION

RT-PCR was employed to detect the expression of a delta opioid receptor in rat A-10 cells. By using this technique we were able to convert total cytoplasmic RNA into DNA, which was subjected to PCR by using known rat DOR primers. PCR product revealed a DNA fragment of expected size (597 bp oligonucleotide). The isolated DNA fragment was then subjected to cloning and sequencing. The sequencing data for this fragment revealed 100 % homology to the known delta opioid receptor isolated from rat brain. Although demonstration of DOR in a cell line is of minimal use, it suggested the presence of DOR in VSM. RT-PCR evaluation of vascular tissue would not be informative since endothelial cell contamination (albeit low-level) is unavoidable and endothelial cells have been demonstrated previously to express DOR (Stefano GB, *et al.*, 1998).

Taken together, this data demonstrated the presence of DOR-mRNA expression in A-10 cells. Since we have demonstrated the presence of a message for DOR expression, the next step is to evaluate the expression of DOR protein. To detect the protein expression, we performed competitive binding studies in rat and human vascular tissue and this study will be discussed in upcoming chapter.



Fig–2. RT-PCR detection of DOR mRNA expression in rat aortic smooth muscle cells. Molecular weight marker (lane 1). PCR products in the presence of forward and reverse primers alone (lane 2 & 3 respectively). PCR products in the presence of forward and reverse primers (lane 4): Note a band of 597 bp. PCR products in the absence of reverse transcriptase (lane 5).

Table–3: **Showing the sequence homology of the cloned DOR fragment with that of MOR and KOR sequences.**

SEQUENCE HOMOLOGY	
Type of Receptor	% Homology
DOR	100
MOR	33.7
KOR	21.4

CHAPTER TWO:

PHARMACOLOGICAL EVALUATION OF DOR BINDING SITE

INTRODUCTION

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Chapter Two:

Pharmacological Evaluation of DOR Binding Site

INTRODUCTION

A number of laboratories have characterized the binding profiles of delta opioid receptor in brain and immune cells. The most selective agonist radioligands for the specific labeling of delta opioid receptors are tritiated or radioiodinated derivatives of DPDPE, deltorphins-I and II and DAMA [D-Ala^2, Met^5-enkephalinamide]. DOR has been reported in pharmacological studies by Jiang Q, *et al.*, and Sofuoglu M, *et al.*, in 1991. They first reported the basis of differential blockade of the action of delta opioid receptor agonists by different delta-selective agonists. Subsequently, studies with NG108-15 cells and brain membranes confirmed the existence of DOR subtypes as δ_1 and δ_2 recognition sites (Fang L, *et al.*, 1994, Fowler CJ, *et al.*, 1994).

The first antagonists reported to exhibit significant selectivity for the delta opioid receptors were enkephalin analogues. In 1982 Shaw JS, *et al.*, demonstrated a 30-fold selectivity for this type of receptor over others but it is an antagonist of low potency. It was 1988 when Portoghese PS, *et al.*, reported a highly selective and potent delta opioid receptor antagonist namely naltrindole(NTI), a naltrexone derivative. It is highly selectivity for delta opioid receptors as compared to mu and kappa receptor. Thus in our experiment, two compounds *i.e.*, naltrindole and met-enkephalin were used to determine DOR expression followed by DOR sub-type (Fig-3).

MATERIAL AND METHOD

Animal Preparation: Male Sprague-Dawley (SD) rats (Charles River Lab. Inc., MA) (250-400 g) were kept in an animal facility. They were fed standard rat chow and had free access to water. The animal was sacrificed by asphyxiation with CO_2. The thoracic aorta was immediately removed and placed in physiological saline solution (PSS) buffer (NaCl 130mM; KCl 4.7mM; $MgSO_4$ 1.17mM; KH_2PO_4 1.18mM; $NaHCO_3$ 14.9mM; Dextrose 5.5 mM; EDTA .03mM; $CaCl_2$ 2.5mM., pH~7.4).

Rat thoracic aorta and human IMA (human internal mammary artery was obtained from five volunteer subjects during their elective coronary artery bypass grafting procedures, SUNY medical center., NY), was denuded of endothelium by inserting a 1 mm knife inserted into the lumen, as described previously (Magazine HI, *et al.*, 1994). Tissue was homogenized in 50 volumes of 0.32M sucrose solution at 4°C by Brinkmann Polytron homogenizer (Rexdale, Ontario, Canada). The resulting homogenate was centrifuged at 9000 X g for 10min. The supernatant was removed and the crude pellet was re-homogenized in 30 volumes of sucrose/Tris-Hcl buffer (Sigma Chemical Inc, MO), followed by centrifugation at 9000 X g for 10min. The procedure was repeated once more and the combined supernatant of two extractions was centrifuged at 30,000 X g for 15min. The resulting pellet was washed by centrifugation in 50 volumes of the sucrose/Tris-HCl solution. This pellet was then re-suspended with a Dounce hand-held homogenizer (Kontes

Glass Company, NJ), in 100 volumes of sucrose/Tris-Hcl solution. Extracted membranes were then immediately used for binding assay. To test several conditions, membranes were aliquoted and then incubated with non-radioactive compounds for 10min at 22°C followed by 60min incubation at 4°C with radioactive compound (six different concentrations of opioid ligands were incubated with 1nM of [^{3}H]-DAMA and a curve was generated for inhibition of tritiated DAMA. The concentration, gave 50% inhibition of [^{3}H]-DAMA binding, is reported as IC_{50} value, see table-4). Membranes incubated with met-enkephalin were pretreated with 10μM phosphoramidon to prevent the degradation of met-enkephalin by neutral endopeptidases.

RESULTS

Our competitive binding studies clearly indicated a preference of the receptor for δ-selective opioid receptor agonist (Table-4). The δ-specific agonists: DAMA; deltorphin; and [met]-enkephalin and the δ-specific antagonist, naltrindole, potently displaced the binding of [^{3}H]-DAMA to membranes of rat aortic smooth muscle and human IMA. The delta specific ligands DADLE and DPDPE and the non-selective opioid receptor antagonist, naloxone potently displace binding of [^{3}H]-DAMA to classical δ_1 receptor. In contrast, opioid receptor on aortic smooth muscle was relatively insensitive to these ligands consistent with that of δ_2 receptor subtype. The delta selective agonists: DAMA; Deltorphin 1; and Met-enkephalin potently displaced the binding of ^{3}H-DAMA to aortic smooth muscle (Table-4). In contrast, ^{3}H-DAMA binding was relatively insensitive to displacement by MOR or KOR selective ligands, suggesting predominant DOR expression.

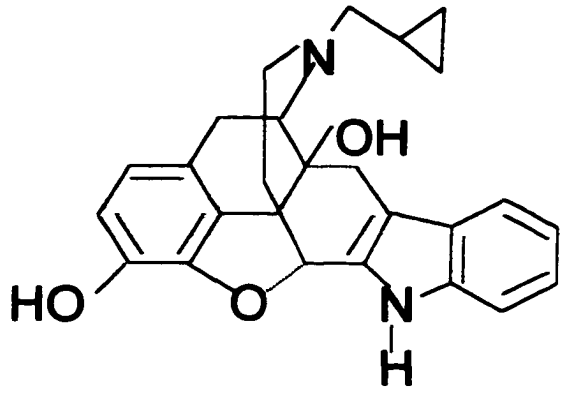
DISCUSSION

DOR binding sites in rat aorta and human IMA were evaluated to confirm that DOR is expressed in arterial preparations of normal tissue and not limited to smooth muscle cell lines. Inhibition of ^{3}H-DAMA binding to membrane homogenates prepared from rat aorta and human IMA that had been denuded of endothelium, were assayed in the presence of opioid receptor subtype-selective agonists and antagonists. These data demonstrate expression of DOR in human and rat arterial preparations. This data is consistent with displacement patterns observed in human and rat endothelial cells and immunocytes of the marine mollusk, *Mytilus edulis*, known to express δ_2 receptors (Stefano GB, *et al.*, 1992).

Taken together, these data suggested the presence of a DOR-like protein expression in human and rat vascular smooth muscle. This study is consistent with our previous data demonstrating the presence of mRNA for DOR in smooth muscle cells.

So far, we have evaluated the presence of RNA and protein expression in vascular smooth muscle. The availability of the commercial anti-DOR antibodies led us to locate DOR expression. We performed immunocytochemical analysis using anti-DOR antisera in A-10 cells to visualize the receptor and this study will be discussed in an up coming chapter.

a) **Naltrindole**



b) **[D-Ala^2]-Met-Enkephalinamide (DAMA)**

Tyr-D-Ala-Gly-Phe-Met-NH_2

Fig –3: Structure of Naltrindole (a). Sequence of DAMA (b)

Table-4. Inhibition of 3H-DAMA binding to vascular smooth Muscle

		IC_{50} (nM)	
Receptor subtype specificity	Ligand	Rat Aorta	Human IMA
Delta agonist	DAMA	0.8 ± 0.0	0.7 ± 0.0
	[D-Ala2] Deltorphin 1	0.7 ± 0.2	0.7 ± 0.1
	[Met] – Enkephalin *	0.9 ± 0.2	0.8 ± 0.1
	DADLE	8.2 ± 1.2	9.1 ± 1.6
	DPDPE	9.1 ± 1.5	9.3 ± 1.8
Mu agonist	DAMGO	12.3 ± 2.3	11.7 ± 2.2
	Dihydromorphine	13.2 ± 3.1	12.7 ± 3.5
	Morphine	13.9 ± 4.2	13.4 ± 4.1
Kappa agonist	Dynorphin (1-17)	16.4 ± 5.8	15.3 ± 3.8
Antagonist	Naloxone	12.8 ± 5.6	7.4 ± 3.2
	Naltrexone	6.1 ± 2.3	6.3 ± 2.7
	Naltrindole	0.8 ± 0.3	0.6 ± 0.0

* preincubation of 10μM phosphoramidon

CHAPTER THREE:

IMMUNOCYTOCHEMICAL ANALYSIS OF DOR EXPRESSION

INTRODUCTION

MATERIAL AND METHOD

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Chapter Three:

Immunocytochemical Analysis of DOR Expression

INTRODUCTION

Distribution patterns of delta opioid receptors have been obtained by two methods: immunocytochemical analysis with specific antiserum and autoradiographic techniques using selective radioligands. Most of the work has been demonstrated in central nervous system (Waksman G, *et al.*, 1986; Mansour A, *et al.*, 1988; Renda T, *et al.*, 1993). We employed immunocytochemical analysis for determining the DOR-distribution pattern in vascular smooth muscle. Antibodies generated against selective portion of the delta opioid receptor amino acid sequence were used to localize the receptor. We used DAMA as DOR-agonist and Naltrindole as DOR-antagonist due to their high selectivity for δ_2 subtype as suggested by our pharmacological studies.

MATERIAL AND METHOD

A-10 cells were plated on a coverslip (Fisher Scientific, TX), and placed in a petri dish in DMEM for 48 hr (see chapter one for cell culturing technique). Following incubation immunocytochemistry was performed at 4°C. Cells were fixed in 4% formaldehyde for 15 min and then washed with PBS (NaCl 137mM; KCl 2.7mM; $Na_2HPO_4.7H_2O$ 4.3mM; KH_2PO_4 1.4mM., pH~7.4) Primary anti-rat delta opioid receptor specific antiserum (1:1000 dilution in PBS) (Chemicon Int. Inc. CA) was added for an hr. Unbound antibodies were removed with several washes of PBS. FITC-labeled goat anti-rabbit IgG was then added (1:200 dilution in PBS) to cells for an hr incubation (Chemicon Int. Inc. CA). Cells were then washed 5 times with PBS. The coverslips were then mounted on to microscope slide with a drop of fluoromount-G (Fisher Scientific, TX). Finally, an immunofluorescence confocal micrograph was obtained using the Meridian confocal microscope. Laser setting was obtained according to the manual provided by the company for a visible imaging analysis (pinhole: 1600 μm; laser power: 600mw; scan strength: 5%; PMTs: 30%).

For evaluation of specificity, the experiment was performed as described above except that the anti-DOR antisera was preincubated for 30 min with 10μM concentration of a DOR N-terminal peptide, NH_3-LVPSARAELQSSPLV-NH_2, used as an immunogen to produce the anti-DOR antiserum or an irrelevant control peptide (10μM) corresponding to the N-

terminus of the thrombin receptor, NH_3-SFLLRNPNDKYEPF-NH_2. Peptides were synthesized in our laboratory using rink resin on the Advanced ChemTech peptide synthesizer Model 90 using standard solid phase peptide synthesis protocol (Magazine HI, *et al.*, 1996). Peptides were cleaved with a cocktail of 90% trifluroacetic acid, 5% 1,2 ethanedithiol, 4% water and 1% thioanisol. Peptides were further purified using column chromatography. 40% acetonitril was used to extract the peptide from the column.

Statistical analysis was obtained by analyzing the average fluorescence per cell. Then average fluorescence values of all the cells in each condition were plotted after subtracting background fluorescence (fluorescence of secondary antibody alone) (see Fig-5).

RESULT

The receptors were localized on the rat A-10 cells, using anti-DOR-specific polyclonal antisera and visualized following incubation with FITC-conjugated secondary antibodies, using quantitative fluorescent confocal microscopy (Fig-4). Intense immunofluorescence was observed in A-10 cells treated with anti-DOR and secondary antibody (Fig-4b) whereas no significant fluorescence was observed in samples treated with secondary antibody alone (Fig-4a). Immunofluorescence was abolished by preincubation of anti-DOR with the N-terminal blocking peptide used to generate the antiserum (Fig-4c) whereas preincubation with TRAP-14, an irrelevant, control peptide had no effect on specific immunolabelling (Fig-4d).

Statistical Analysis of anti-DOR immunofluorescence in rat A-10 cells was also evaluated (Fig–5). Specific anti-DOR Immunofluorescence was significantly reduced by preincubation with anti-DOR N-terminal blocking peptide whereas pretreatment with peptide of scrambled sequence had no effect (control).

DISCUSSION

This study provides the first direct evidence for DOR localization in rat vascular smooth muscle. Our immunocytochemical analysis demonstrated anti-DOR immunoreactivity in A-10 cells (Figure-4) confirming expression of DOR or a DOR-like protein. To date, molecular and immunohistochemical techniques have failed to discriminate between the δ_1 and δ_2 receptor sites. However, these data coupled to our competitive binding studies suggested the presence of a δ_2 subtype.

Taken together this data suggested the presence of a delta opioid receptor expression in vascular smooth muscle, which is consistent with our previous studies, demonstrated mRNA and protein expression for this receptor. These data led us to move one step forward and evaluate the functional aspect of the receptor. To examine the receptor function we evaluated the signaling mechanism of DOR and this will be discussed in chapter four.

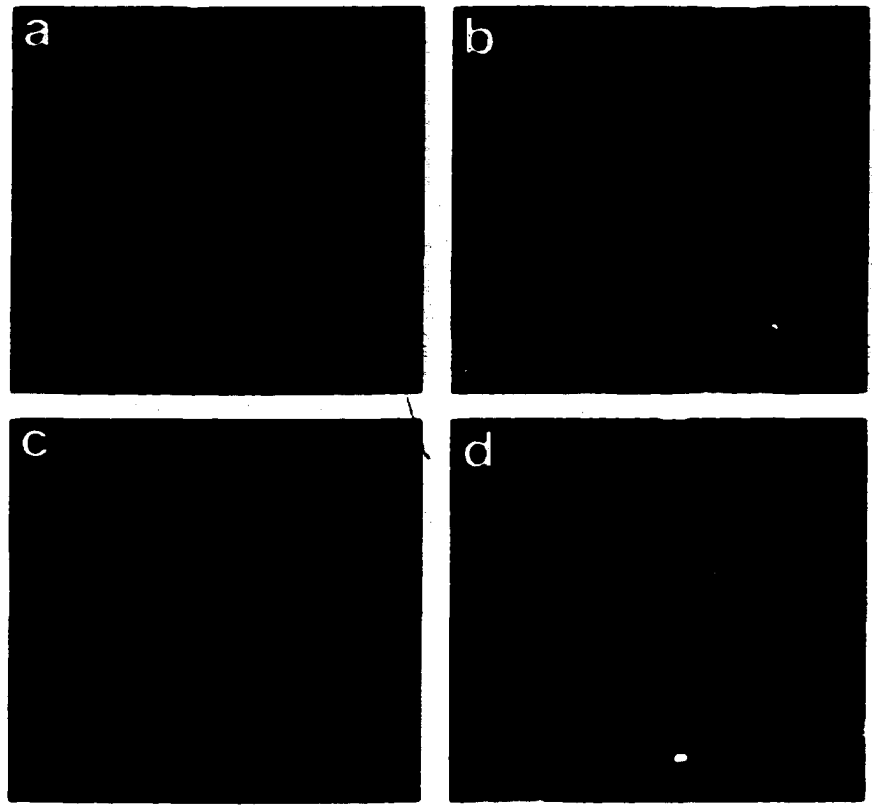


Figure –4: Expression of DOR in A-10 cells. Immunofluorescence of A-10 cells treated with FITC-conjugated antibody without primary antibody (A) is significantly lower than those pretreated with primary DOR specific antiserum (b). Preincubation of DOR primary antibodies with the rat DOR N-terminal peptide used as an immunogen to produce the antisera eliminated the DOR immunolabelling (c) whereas preincubation with a scrambled control peptide had no effect (d).

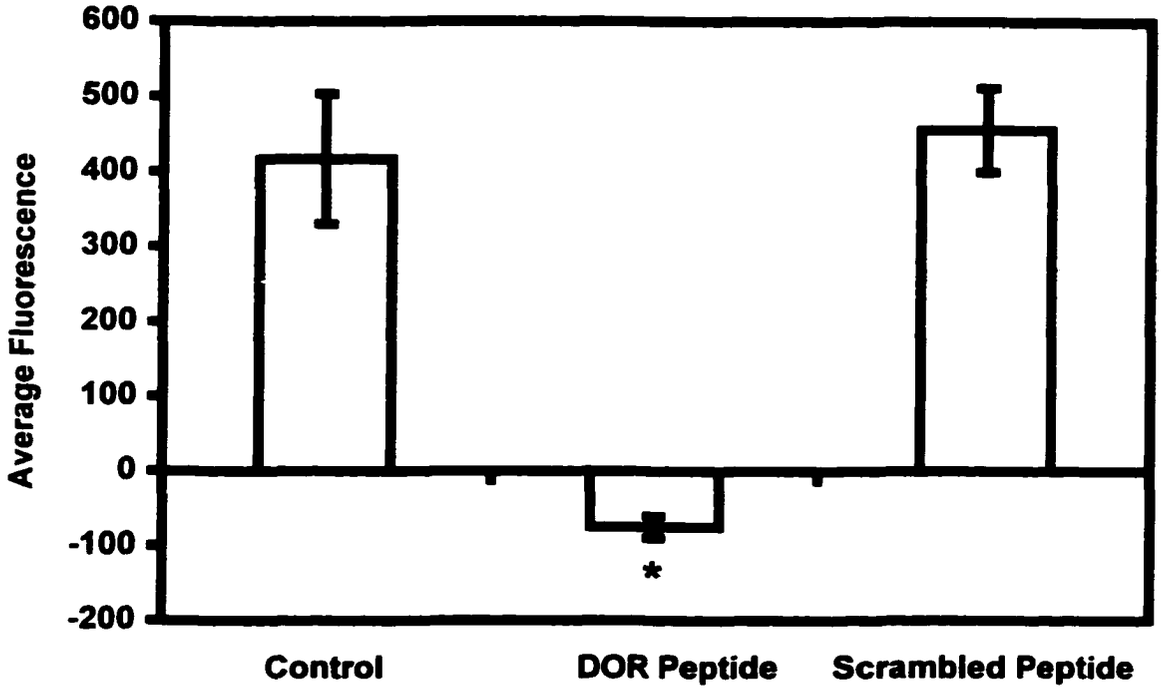


Figure-5: Statistical Analysis of anti-DOR immunofluorescence in rat A-10 cells. Fluorescence of A-10 cells treated with primary rat anti DOR antisera followed by FITC-labeled rabbit anti rat secondary antisera was reduced significantly in A-10 cells treated secondary antisera alone. Specific anti-DOR fluorescence was abrogated by preincubation with a DOR N-terminal blocking peptide. Specific anti-DOR fluorescence was not altered by preincubation with a scrambled peptide (control). Data represents fluorescence – background (secondary antisera alone). Five experiments were performed (*P< 0.001 as determined by ANOVA).

CHAPTER FOUR:

SECOND MESSENGER SYSTEMS COUPLED TO DOR

INTRODCUTION

MATERIAL AND METHODS

RESULTS

a. Change in Membrane Potential

b. Western Blot

c. Change in Intracellular Calcium Release

CONCLUSION

Chapter Four:

Second Messenger Systems Coupled to DOR

INTRODUCTION

Though the role of opioid receptors for their activation of K^+ channel, and mobilization of calcium from intracellular stores is well documented for neurons and brain cells, no significant work has been done to determine the signaling mechanism in smooth muscle. Among the first few reports was the study of Werz MA, *et al.*, in 1983 they demonstrated that delta and mu selective opioid peptides reduce calcium-dependent action potential duration by increasing potassium conductance in dorsal root ganglion. One year later this group reported that dynorphin reduces calcium-dependent action potential duration by decreasing voltage-dependent calcium conductance in mouse dorsal root ganglion. In 1987 North RA, *et al.*, reported that delta opioid receptors are coupled to potassium channels in guinea pig submucous plexus and rat nucleus locus coeruleus. In 1989 Miyaki M and his co-workers demonstrated the direct coupling of mu opioid receptor with potassium channels through local intermediary action of a GTP binding protein in rat locus ceruleus neurons. Recently, Chiappinelli VA, *et al.*, (1993) reported a major opioid-induced depolarizing effect associated with a decrease in resistance in the nerve terminals of Edinger-Westphal neurons. Fan SF, *et al.*, in 1993 demonstrated that lower concentrations of opioids decreased voltage-

dependent outward K^+ currents during step depolarization and this effect is naloxone reversible.

It has recently been recognized that tyrosine kinases play an important role in regulation of smooth muscle contraction. Several tyrosine kinase inhibitors including genistein suppress agonist-induced contraction in smooth muscle (Di Salvo J, *et al.*, 1993; Hatakeyama N, *et al.*, 1996). It has been demonstrated that cellular depolarization is associated with tyrosine phosphorylation in human bronchial smooth muscle and vascular smooth muscle cells (Liu H, *et al.*, 1997; Liu CY, *et al.*, 1996). Tyrosine phosphorylation is also known to couple to intracellular calcium release and contraction in smooth muscle (Di Salvo JD, *et al.*, 1997; Liu CY, *et al.*, 1996; Cortijo J, *et al.*, 1997). Rapidly emerging data in support of this hypothesis lead us to look for these phenomena in our system.

To elucidate DOR receptor function, we examined the capacity of vascular smooth muscle DOR to alter membrane potential and the possible involvement of tyrosine kinases and intracellular calcium.

MATERIAL AND METHOD

Membrane Potential: A-10 cells were grown in a coverglass chamber (Nalge Nunc Inc., IL) for 48 hrs (see chapter one for cell-culturing technique). Subconfluent monolayers were then washed and equilibrated in Hanks balanced salt solution (HBSS) (KCl 5.4mM; Na_2HPO_4 0.3mM; KH_2PO_4 0.4mM; $NaHCO_3$ 4.2mM; $CaCl_2$ 1.3mM; $MgCl_2$ 0.5mM; $MgSO_4$ 0.6mM; NaCl 137mM; D-Glucose 5.6mM., pH~7.4), containing 1μM of bis (1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4$), an anionic potential sensitive fluorescent dye (Molecular Probes Inc., OR). Following incubation, the cells were imaged in the continuing presence of extracellular $DiBAC_4$ using 488 nm excitation. In response to drug, membrane depolarizes and $DiBAC_4$ enters the cell. Whereupon binding of this dye to intracellular protein molecules increases the $DiBAC_4$ fluorescence. To measure the correlation between increase in fluorescence and change in membrane potential, a kinetic plot of normalized $DiBAC_4$ fluorescence during stepped-depolarization of smooth muscle cells by incremental addition of K^+ from 1.25 to 50mM was made. An increase of 1% in fluorescence resulted in an increase of 2 mV in membrane potential. This method is based upon the previously published studies (Cornfield DN, *et al.*, 1994). Laser-setting was adjusted according to the manual provided by the company for visible imaging analysis (pinhole: 1600 μm; laser power: 600mw; scan strength: 5%; PMTs: 30%).

To examine the involvement of tyrosine kinases, similar experiment was performed with cells pre-incubated with 1μM vanadate (a tyrosine phosphatase inhibitor) / 10μM erbstatin (a tyrosine kinase inhibitor), the concentrations previously shown to provide maximal effect.

Western Blot Analysis: Four rats were sacrificed and thoracic aortas were removed and denuded of endothelium as previously described in chapter two (Magazine HI, *et al.*, 1994). The experimental conditions are shown in Fig-10. Two of the aortas were incubated with 1μM vanadate and 10μM erbstatin for 20 minutes in DMEM prior to the addition of DAMA. After drug treatments tissues were homogenized in teflon glass homogenizer (Kontess glass co. vineland, NJ) in boiling lysis buffer. The homogenates were centrifuged at 16000 X g for 5 min and supernatants were collected. Protein concentration in each sample was determined by using BCA kit (Pierce., Rockford, IL) (Fig-9).

100 μg protein was loaded in each well in a 10% SDS-PAGE. Proteins were transferred on to a nitrocellulose membrane (Amersham, Life Science Ltd., IL) by semi-dry electro-blotting (C.B.S Scientific Inc. CA., model # EBU 400). All incubations and washings were done on an orbital shaker at room temperature unless otherwise mentioned. Membrane was then blocked by 6% nonfat dry milk at 4^0C for overnight. Next day, membrane was washed in Tris-buffered saline containing 0.5% Tween-20 (TBS-T), (NaCl 137mM; Tris-HCl 20mM., pH~7.6) and incubated in the presence of anti-phosphotyrosine

antiserum (1:1000 in TBS-T), (ECF Western blotting kit. Amersham, Life Science Ltd., IL) for an hour and then rinsed. Secondary antiserum was added (anti-rabbit IgG) for an hour at room temperature in TBS-T. Membrane was then washed and further incubated in AP-conjugate for an hour and then thoroughly rinsed. Membrane was incubated with ECF substrate for 20 minutes and then dried. Bands were visualized in blue fluorescence by using phosphoimaging analysis (Molecular Dynamics STORM Imaging System, CA).

Intracellular Calcium Release: A-10 Cells were grown in 2-Chambered coverglass (Nalge Nunc Inc., IL). On the achievement of 70% confluency cells were washed with PBS and loaded with 0.5 μg/ml of Indo-1 AM (Molecular Probe Inc., OR), for 15 min. Cells were then washed 5-6 times with PBS and accumulation of Ca^{2+} was measured in a cell within a single field of vision by Confocal Microscopy. Laser setting was adjusted according to the manual provided by the company for UV imaging analysis (pinhole: 225 μm; laser power: 30mw; scan strength: 5%; PMTs: 35%). Two- detectors were used, one for bound calcium fluorescence and the other for unbound calcium fluorescence.

To examine the source of increase in intracellular calcium, experiments were performed in Ca^{2+} deficient buffer (containing 2mM EGTA) and in the presence of ryanodyne (RBI., MA). A-10 cells were preincubated with 1μM ryanodyne for 20 minutes prior experiment.

RESULT

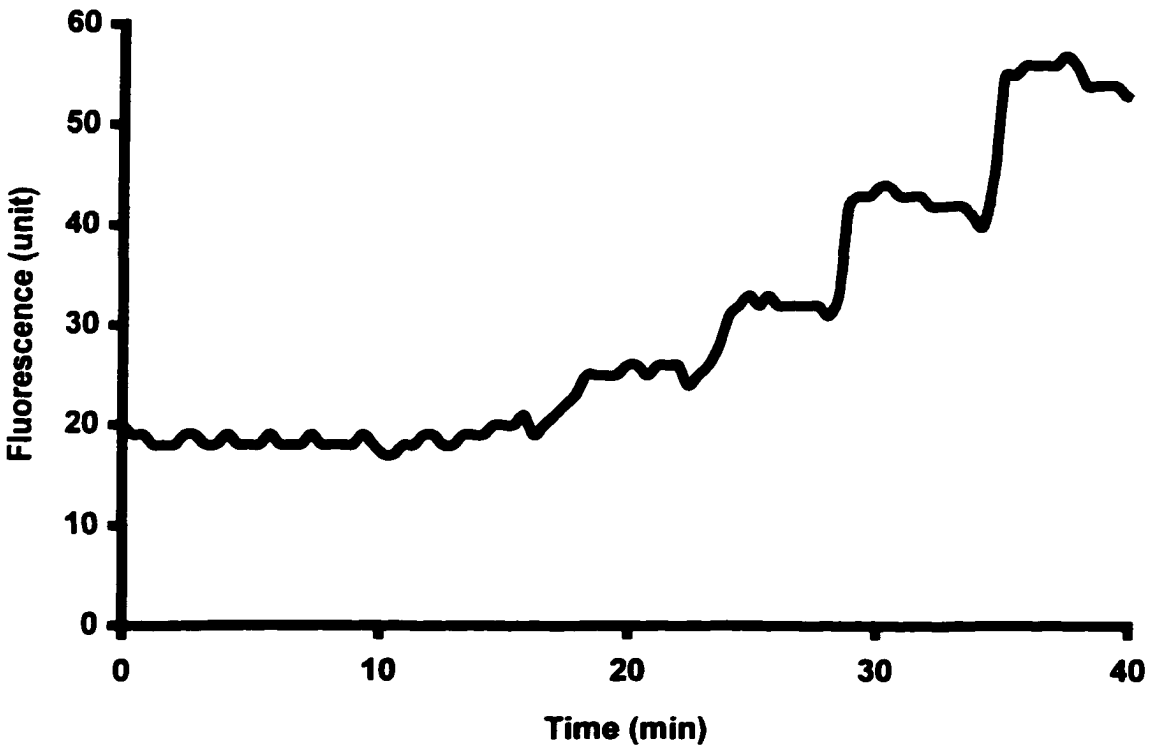
Membrane potential: Opioid receptors, including delta, are well known for their capacity to alter voltage-gated calcium channels (Werz MA, *et al.*, 1984; Surprenant A, *et al.*, 1990; Taussig R, *et al.*, 1992; Jin W, *et al.*, 1994) and to activate K^+ currents (Werz MA, *et al.*, 1983; North RA, *et al.*, 1987; Miyake MJ, *et al.*, 1989; Buzas B, *et al.*, 1998). Treatment of A-10 cells with DAMA triggered a rapid membrane depolarization of 11.6 mV that was abrogated in the presence of naltrindole and erbstatin. Importantly, preincubation with vanadate (a tyrosine phosphatase inhibitor), strongly enhanced the response suggesting the involvement of tyrosine kinases (see Fig-6, 7 & 8).

Western blot: Based upon our membrane depolarization studies and the fact that tyrosine phosphorylation is coupled to membrane depolarization and intracellular calcium release in smooth muscle (Di Salvo J, *et al.*, 1997), we examined the levels of tyrosine phosphorylation in rat aortic tissue in a number of conditions (Fig-10). Western blot analysis with anti-phosphotyrosine antiserum showed a tyrosine phosphorylated protein of ~ 200 kDa in control conditions. This was markedly increased by treatment with DAMA and further enhanced by preincubation with vanadate. This phosphorylation was abrogated by pretreatment with erbstatin (Fig-10).

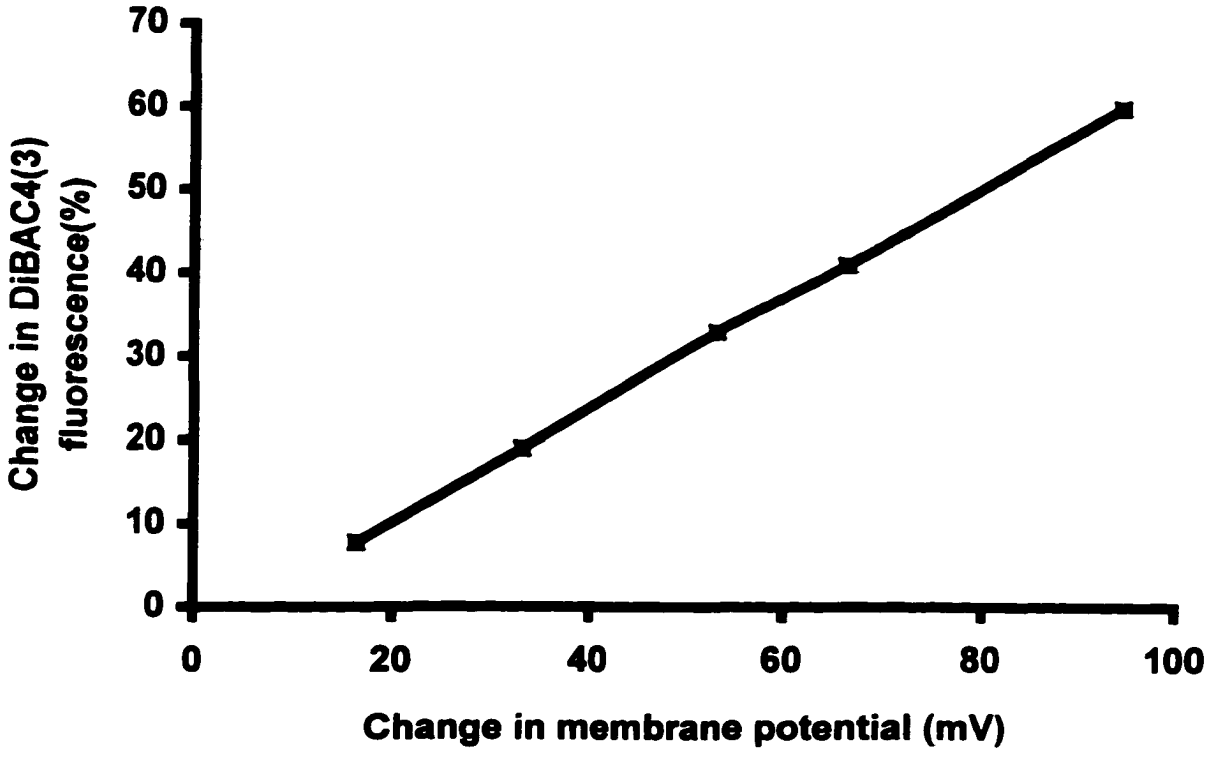
Intracellular calcium release: Delta opioid receptor-mediated depolarization induces an increase in intracellular Ca^{2+} in NG108-15 cells (Buzas B, *et al.*, 1998). Additionally, opioids are known to mobilize calcium from intracellular stores (Jin W, *et al.*, 1994). To investigate the activity of these mechanisms in rat aortic smooth muscle, A-10, cells were labeled with the Ca^{2+} sensitive dye, Indo-1 AM, and changes in intracellular free Ca^{2+} were evaluated by quantitative confocal microscopy (Fig-11 & 12). Treatment of A-10 cells with DAMA resulted in an 80 nM increase in intracellular calcium that was inhibited in the presence of naltrindole. However, the response of DAMA in the absence of extracellular calcium, though reduced, but not significantly abrogated it, suggesting the release of calcium mainly from intracellular stores. Importantly, pretreatment with ryanodyne (a sarcoplasmic reticulum Ca^{2+} transport blocker) abrogated DAMA-induced Ca^{2+} mobilization (Fig-13), consistent with Ca^{2+} mobilization from the sarcoplasmic reticulum (Chavis P, *et al.*, 1996; Tanaka K, *et al.*, 1998).

DISCUSSION

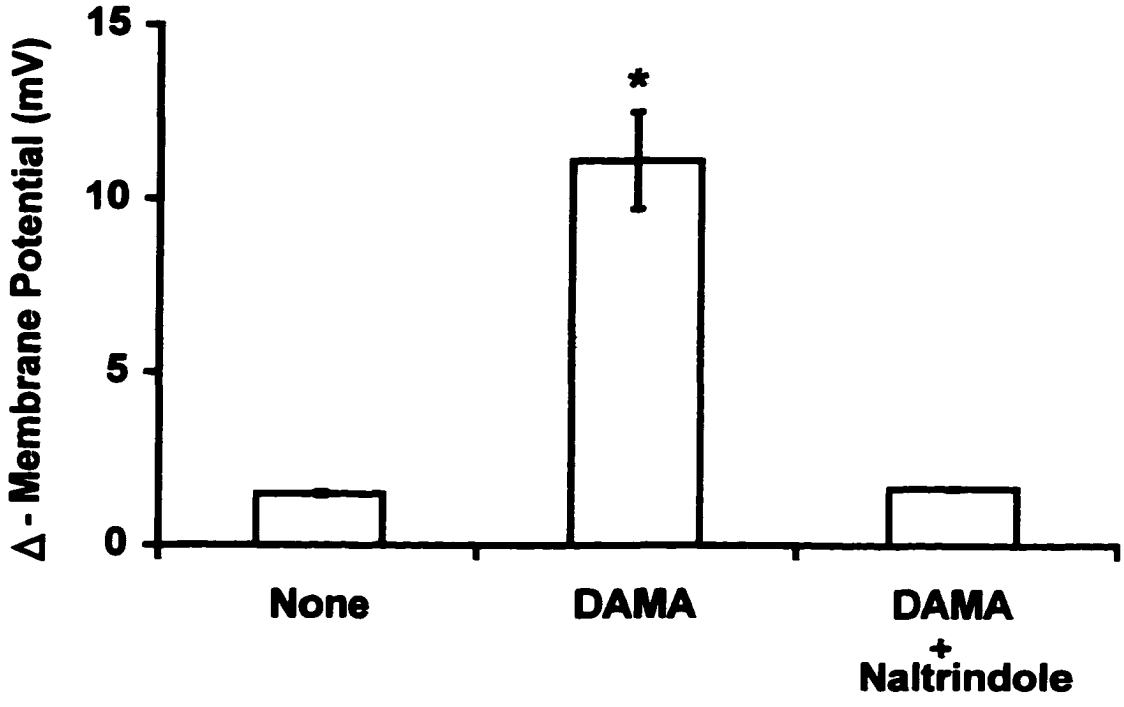
Based upon the signaling studies, we hypothesize that delta opioid receptor activation causes membrane depolarization and intracellular calcium release, which is associated with tyrosine phosphorylation. Membrane depolarization in vascular smooth muscle has been demonstrated previously (Post JM, *et al.*, 1992; Yuan XJ, *et al.*, 1993). Previous studies have demonstrated that blocking of voltage-gated K^+ channels cause subsequent membrane depolarization. Membrane depolarization lead to an increase in intracellular calcium (Cornfield DN, *et al.*, 1994) in pulmonary artery smooth muscle cells. The fact that 11.6 mV change in membrane potential is sufficient to cause release of calcium from intracellular stores (Cornfield DN, *et al.*, 1994) suggests that depolarization may be upstream of calcium release. Ryanodyne-sensitive stores appear to be the main source of this calcium release. Ryanodyne-sensitive release of calcium independent of, IP_3-mediated pathway has been demonstrated by Allouche S 96, for a delta opioid receptor in neuroblastoma cell line SK-N-BE.



Fig–6: Kinetic plot of normalized $DiBAC_4$ fluorescence in A-10 cells. Stepped depolarization of smooth muscle cells by incremental addition of K^+ from 1.25mM to 50mM was evaluated.



Fig–7: Evaluation of membrane potential. The percent increase in A-10 cells $DiBAC_4$ fluorescence vs. the change in membrane potential calculated using the Nernst equation (E_K^+=-59 log [K^+_{in}/K^+_{out}], assuming K^+_{in} = 100mM).



Figure–8: The effect of DAMA on $DiBAC_4$ fluorescence in VSMC. Addition of 1μM DAMA induced 5.8% increase in fluorescence. This change in fluorescence corresponds to 11.6 mV change in membrane potential. VSMC incubated with 10μM Naltrindole (DOR-selective antagonist) for 20 min prior to DAMA addition blocked the response. Preincubation of VSMC with 1μM Vanadate (a tyrosine phosphatase inhibitor) significantly augmented the response to DAMA whereas preincubation with 10μM Erbstatin (a tyrosine kinase inhibitor) impaired the response markedly (n=8, *P<0.001 as determined by ANOVA).

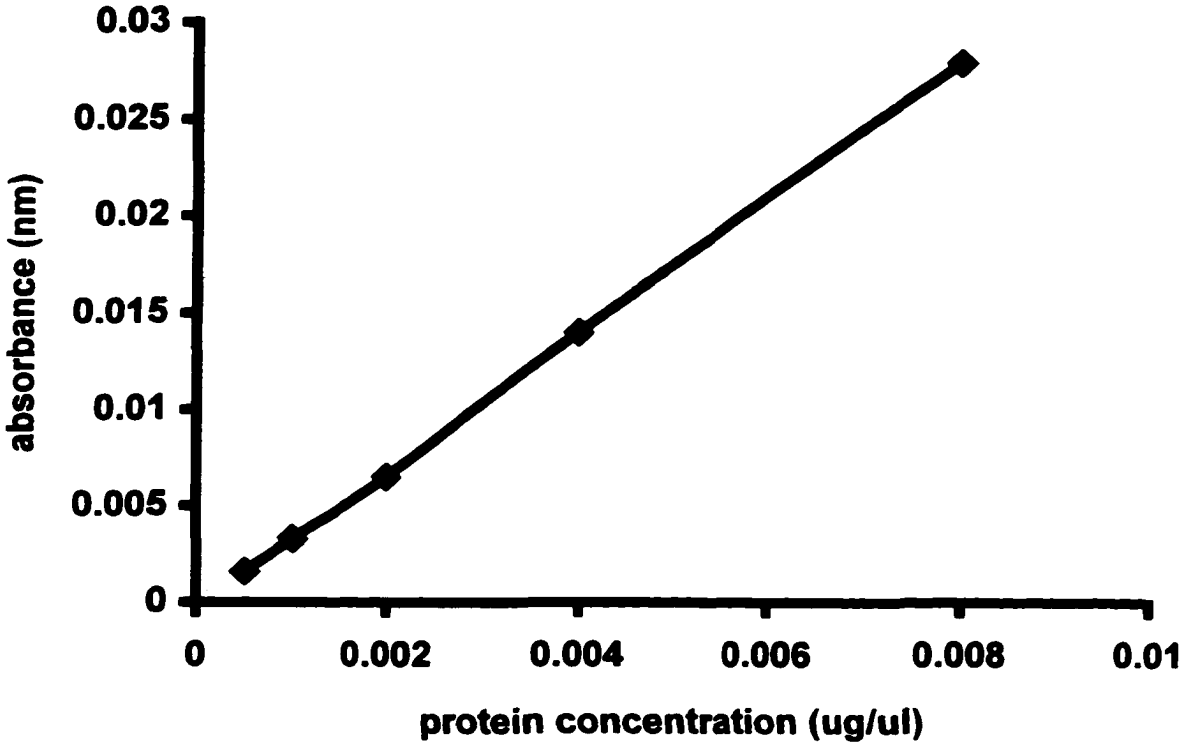
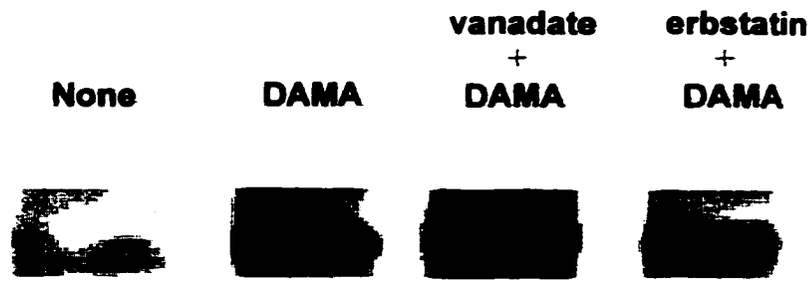
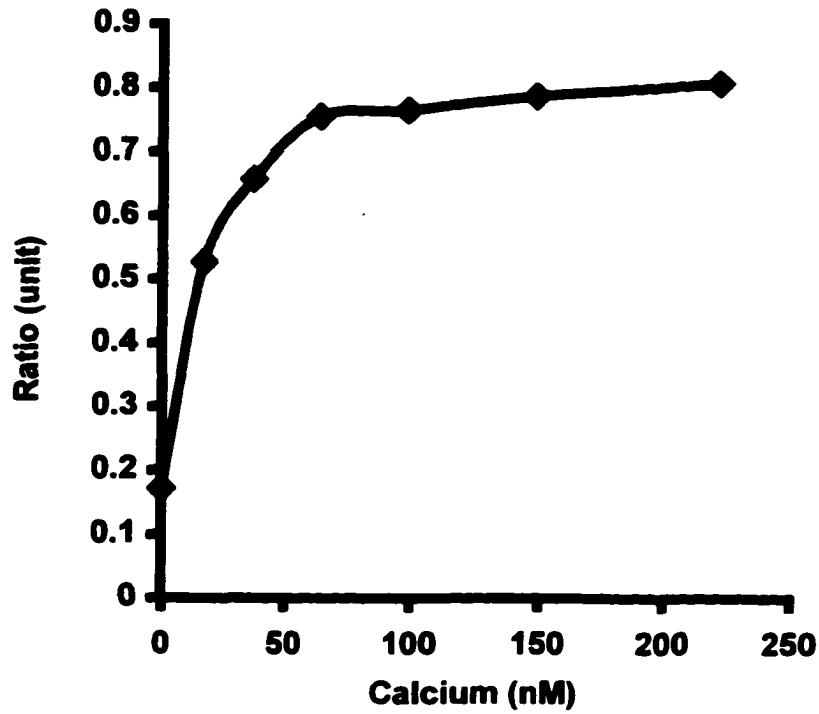


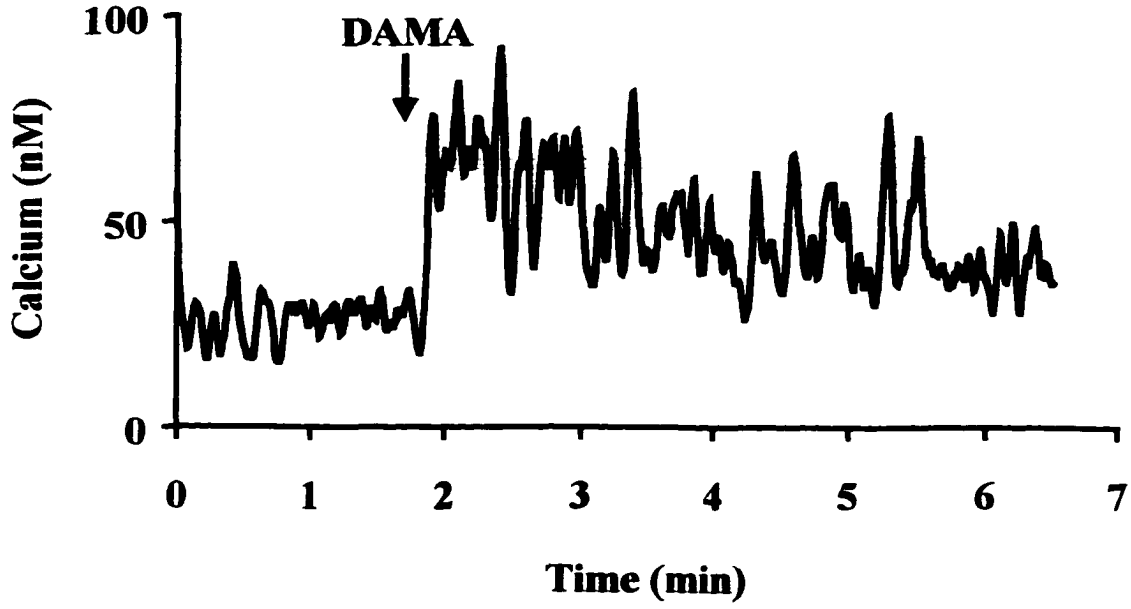
Fig-9: Standard curve to determine the amount of protein. Serial dilutions of standard (BSA; 0.0005, 0.001, 0.002, .004, .008 µg/µl) was made and the absorbance of each standard and samples was measured at 562nm in spectrophotometer. Absorbance of standards was plotted against concentrations to determine the amount of protein in unknown sample.



Fig–10: Effect of vanadate and erbstatin on DAMA-induced protein tyrosine phosphorylation. Denuded rat aortic tissue was pretreated with 1μM vanadate and 10μM erbstatin before extracting the membranes. Tyrosine phosphorylation in untreated tissue (lane–1). DAMA caused an increase in phosphorylation which was further augmented by vanadate (lane 2 & 3 respectively). Pretreatment with erbstatin blocked DAMA-induced phosphorylation (lane 4).



Fig–11: Standard curve for determination of fluorescence-based calcium. The ratios calculated from the fluorescent signals (detected at 405 nm and 485 nm) are plotted against the known calcium concentrations in the experimental buffer. The standard curve was obtained by using the ratiometric probe Indo-1 with PMT values identical to the one used in the experiment. The calculated ratio of calcium chelated Indo-1 to free Indo-1 was later compared with the standard curve in order to generate calcium concentration within the cell.



Fig–12: Changes in intracellular calcium concentration in response to 1μM DAMA. A representative tracing of DAMA-induced calcium release is shown in A-10 cells loaded with Indo-1 AM (see material and method). Upon addition of DAMA, an increase in fluorescence was achieved which corresponds to ~80 nM increase in intracellular calcium.

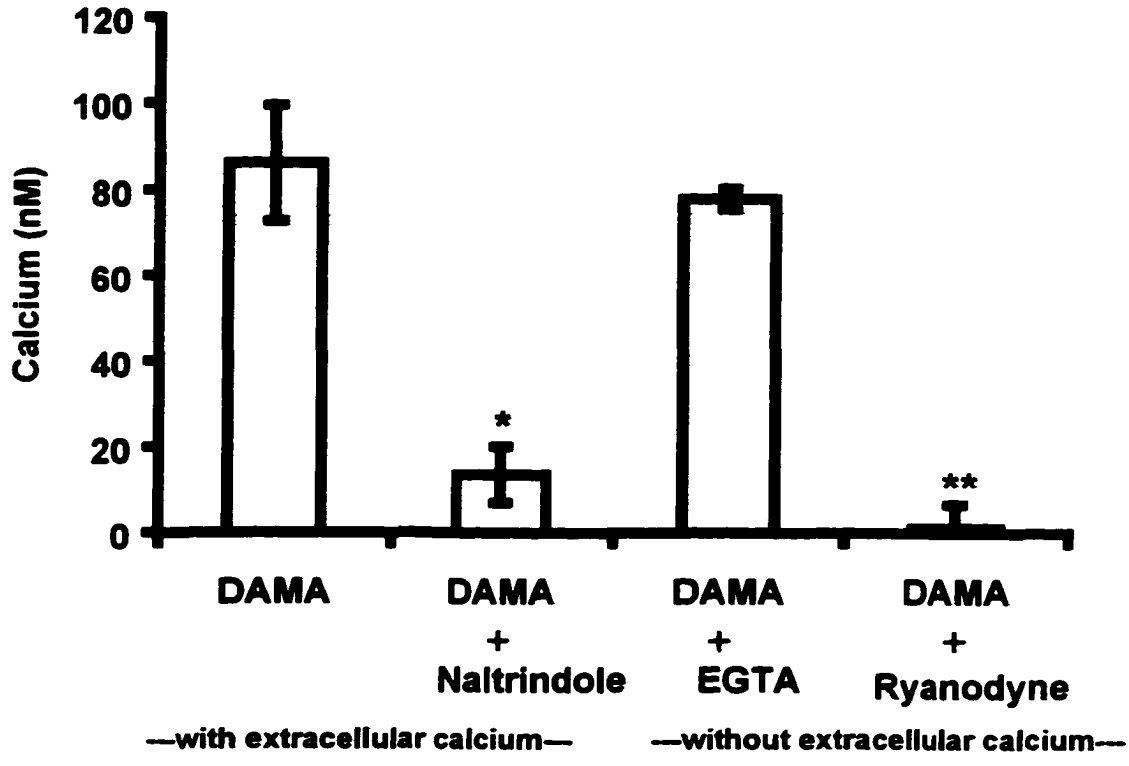


Fig-13: Changes in peak calcium release upon stimulation of DAMA. Preincubation of 10μM naltrindole or 1μM ryanodyne abrogated DAMA-mediated intracellular calcium increase (n=5, *P<0.01 and **P<0.001, student's t-test). In contrast, level of intracellular calcium accumulation was not significantly altered in the absence of extracellular calcium, suggesting the release of calcium from intracellular stores.

CHAPTER FIVE:

PHYSIOLOGICAL RESPONSE

INTRODUCTION

MATERIAL AND METHOD

RESULT

DISCUSSION

Chapter Five:

Physiological Response

INTRODUCTION

It has been demonstrated that agonists of the mu and delta-opioid receptor, as morphine and enkephalins can alter vascular tone when administered *in vivo* (Wahl M, *et al.*, 1985). Such effects were thought to be indirect and mediated by opiate / opioid-peptide induced neurotransmitter release by sympathetic and parasympathetic nerve endings. However, a direct effect of opioids on vascular tone has been demonstrated by limited studies. Opioid receptor activation induced vasodilation of isolated feline middle cerebral artery and arterioles of the hamster cheek pouch while vasoconstriction has been reported for rat portal vein exposed to opioids (Hanko Jh, *et al.*, 1978; Wong TM, *et al.*, 1981 and Yamamoto Y, *et al.*, 1984). Opioids have also been reported to interact with other circulating vasoactive agents. Indeed, Ruth JA, *et al.*, 1984 reported that Leu-enkephalin inhibits norepinephrine-induced contraction of rat aorta.

Direct effects of opioids are difficult to demonstrate in vascular smooth muscle due to the retention of functional nerve fibers in the preparation. The aorta is known to be poorly innervated. Thus, nerve endings are unlikely to contribute to responses observed. However, we preincubated the rings with the neurotoxin, tetrodotoxin, to abrogate any residual nerve activity to ensure

that any changes in vascular tone observed, were a direct effect of DAMA on vascular smooth muscle.

In this chapter we evaluate the functional coupling of delta opioid receptor activation to vascular contraction.

MATERIAL AND METHOD

Male Sprague-Dawley rats, 3-6 months of age, were sacrificed (as described in chapter two), followed by removal of aorta for evaluation of developed isometric tension (Magazine HI, *et al., 1994*). The vessel was placed in physiological salt solution (PSS), and excess connective tissue was removed. The preparation was cut into 3mm rings, mounted on metal tissue holder, and placed in a 7ml tissue bath (Kent Scientific Corp.) containing aerated (95% O_2, 5% CO_2) PSS buffer maintained at 37°C. Maximal contraction of the rings was assessed by measurement of contraction to 1M KCl. Changes in vascular tone were detected by computer-interfaced force transducers (Kent Scientific Corp.) set at a sampling rate of 6 recordings/min. All the drugs were purchased from RBI., MA, unless otherwise mentioned. 1μM tetrodotoxin was added in all the baths to block the nerve responses (tetrodotoxin is neurotoxin that blocks Na^+ channel in neuron and has no effect on vascular tension). Rings were then preincubated with 10μM phosphoramidon to prevent degradation of DAMA by neutral endopeptidases. Contraction in response to cumulative addition of DAMA (10^{-8} - $10^{-4.5}$ M) was measured. In order to examine the effect of inctracellular Ca^{2+} stores, rings were preincubated with 1μM ryanodyne, an agent that depletes RyR-gated sarcoplasmic reticulum store of calcium.

RESULT

Contraction of vascular rings to 1μM DAMA was evaluated using rings of rat aorta. Contraction induced by DAMA (1μM) was 7.73 ± 2.57% of KCl maximum (Fig-14). DAMA induced contraction was reduced to 1.49 ± 0.42 % in the presence of 1nM naltrindole (Fig-16). Use of naltrindole at concentrations greater than 1 nM resulted in modest contraction of the aortic rings, consistent with the known partial agonist properties of opioid antagonists when used at high concentrations (Fang X, *et al.*, 1997). A cumulative dose response curve to DAMA was also measured (10^{-8} to 30 x10^{-6} M,) to evaluate contractile potency. DAMA failed to induce contraction at concentrations less than 10x10^{-9} M. Initiating the response with a dose of 30x10^{-9}, increasing doses of DAMA, stimulated increased contraction (Fig–15). To evaluate the role of extracellular Ca^{2+} in DAMA induced contraction, DAMA-induced contraction was evaluated in Ca^{2+} deficient buffer containing 2mM EGTA. DAMA-induced contraction was reduced by approximately 50% to 3.96 ± 1.09% in the absence of extracellular Ca^{2+} (See Fig-17). To evaluate the role of intracellular Ca^{2+} in DAMA-induced contraction, rings were pretreated with the SR Ca^{2+} channel agonist, Ryanodyne. Exposure of cells to ryanodyne results in depletion of SR Ca^{2+} stores, thus inhibiting subsequent RyR mediated responses. Pretreatment of aortic rings with 1μM ryanodyne potentiated DAMA-induced contraction by 2.4 fold (Fig-18). These

observations suggest that both intracellular and extracellular Ca^{2+} sources contribute to DAMA-mediated contraction.

DISCUSSION

To determine the effect of delta opioid receptor activation on vascular smooth muscle tone we measured the effect of DAMA on of rat aortic rings, denuded of endothelium. Addition of 1μM DAMA caused an increase of 7.73 ± 2.57% of KCl max in contraction. In initial experiments, we attempted to block the effects of DAMA using 10μM naltrindole. However, doses of naltrindole greater than 1nM exerted agonistic properties resulting in smooth muscle contraction. We repeated the experiments in aortic rings that had been pretreated with a low dose, 1nM, of naltrindole and were able to abrogate DAMA-mediated contraction. DAMA-induced contraction was reduced by 80% to 1.48 ± 0.42 % of KCl max. Since the K_d value of DAMA is 10-50 fold less than that of naltrindole (K_d of naltrindole=0.04 -0.25nM; K_d of DAMA=1.3nM) and the tissue was preincubated with the antagonist, it is reasonable to assume that low doses naltrindole can saturate all the available binding sites for DOR allowing for the inhibition observed. Indeed others have reported similar inhibition of DOR by low dose naltrindole in brain tissue (Yamamura MS, *et al.*, 1992; Carpenter DO, *et al.*, 1995; Drower EJ, *et al.*, 1993). The contractile response of DAMA was slow developing and lasts 35-60 min, which does not resemble the more rapid *in vitro* response of arterial smooth muscle to neurotransmitters (Duckles SP, *et al.*, 1994). A slow developing contractile response, such as developed by DAMA usually involve subcellular mechanisms such as release of calcium from intracellular stores. Furthermore

the low level of contraction induced by DAMA in the absence of extracellular calcium suggests an important role of Ca^{2+} from both intracellular stores and extracellular fluid in DAMA-mediated contraction.

We hypothesize that RYR-mediated release of calcium may contribute to DAMA-induced increase in intracellular calcium concentration (Rasmussen H, *et al.*, 1990). The amount of calcium released from intracellular stores (approximately 80nM, See Fig-12 & 13) is not enough to achieve the level of DAMA-induced contraction observed (Cellular aspects of smooth muscle function edited by Kao and Carsten, published by Cambridge Univ. Press 1997). In the presence of extracellular calcium the level of DAMA-induced contraction is much greater than in the absence of extracellular calcium. To evaluate the role of intracellular calcium, we used ryanodyne to deplete the sarcoplasmic reticulum (SR) calcium stores. Ryanodyne opens SR Ca^{2+}-release channels but has no direct effect on plasma membrane Ca^{2+} channels (Ashida TJ, *et al.*, 1989). In our experiments, aortic contraction was observed following exposure to ryanodyne suggesting that the SR contained sufficient Ca^{2+} to directly alter smooth muscle tone. Following a return to baseline levels of contraction, DAMA induced a marked increase in vascular contraction relative to that of control aorta that had not been exposed to ryanodyne (Fig-18). These experiments taken together suggest that both intracellular and extracellular Ca^{2+} contribute to DAMA-induced contraction. Exposure of the aorta to ryanodyne allowed depletion of SR Ca^{2+} yet increased contraction

was observed following DAMA exposure. These data suggest the SR released Ca^{2+} is not rapidly eliminated from the cytosol and combines with the extracellular Ca^{2+} stimulated by DAMA to produced an marked increase in vascular contraction. The discreet mechanisms that describe the coupling of DOR to RYR have not yet been elucidated.

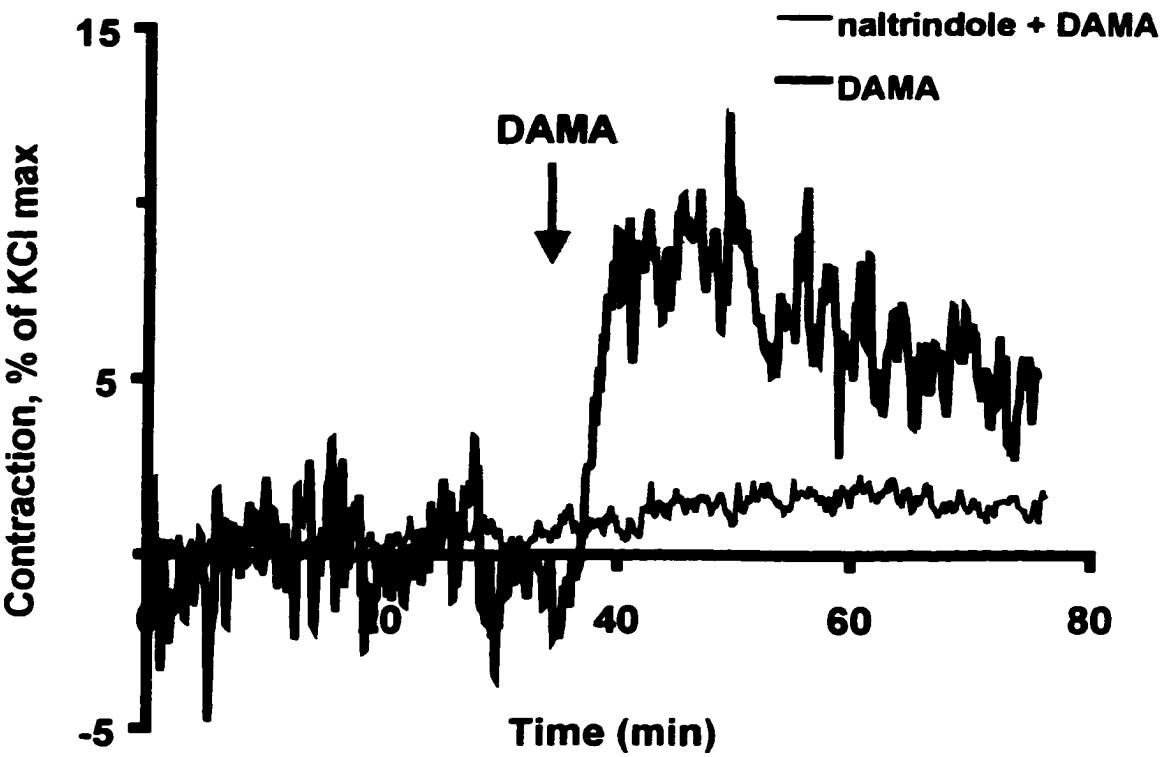


Fig-14: A real time tracing of the contractile response of rat aortic ring to DAMA. Addition of 1μM DAMA cause an increase in contraction by 7.7 ± 2.57 % which lasted for 35-60 min. Preincubation of the ring with 1nM naltrindole abrogated DAMA-mediated contractile response by 80% to 1.48 ± 0.42, suggesting the involvement of δ_2 subtype in DAMA-induced contractile response.

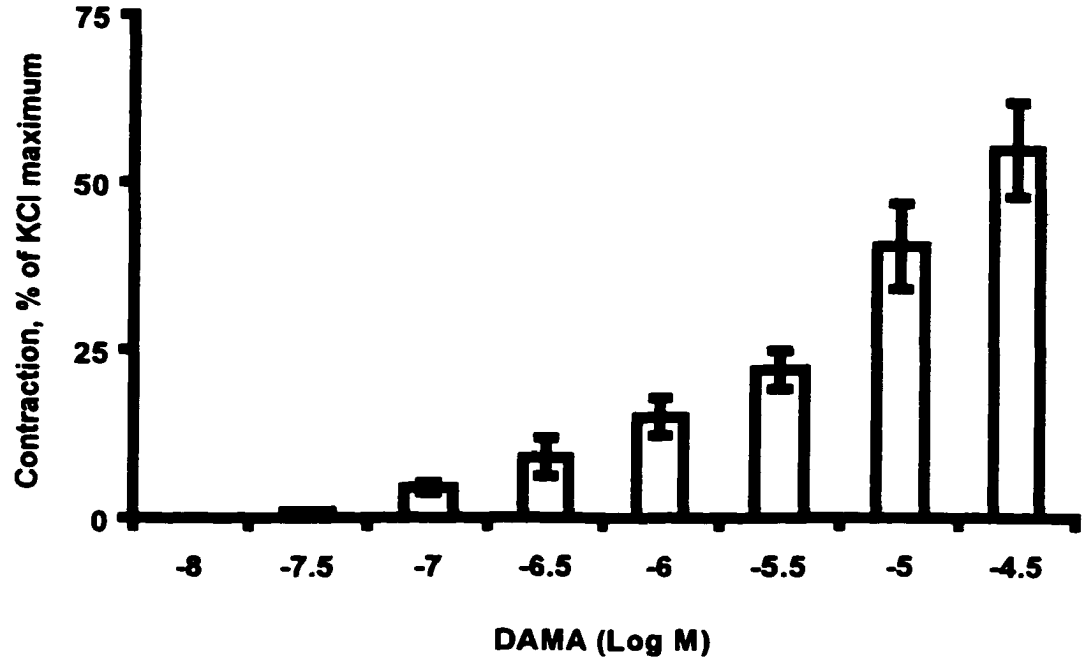


Fig-15: DOR-activation induced contraction in rat aortic rings in response to DAMA dose increment. DAMA doses ranges log –8.0 to –4.5 M was added with 0.5 dose increment after recording the peak response of prior dose. The increase in concentration of DAMA augmented the contractile response of the ring suggesting a direct relationship of dose with that of contraction. This is a representative of n=3 experiments.

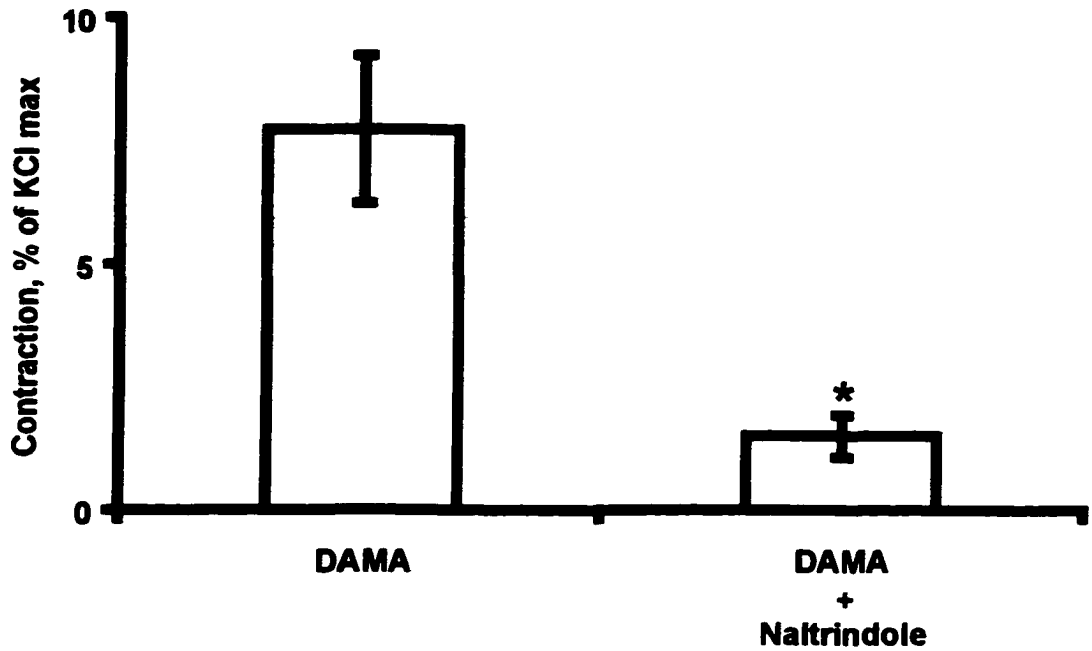


Fig-16: Effect of Naltrindole-preincubation on DAMA-mediated contractile response. Preincubation of the ring with 1nM naltrindole abrogated DAMA-mediated contraction by 80% suggested that δ_2 subtype is responsible for DAMA-induced contractile response. This data was obtained in conjunction with Jose D. Murga. (n=12; *P<0.001, student's t-test).

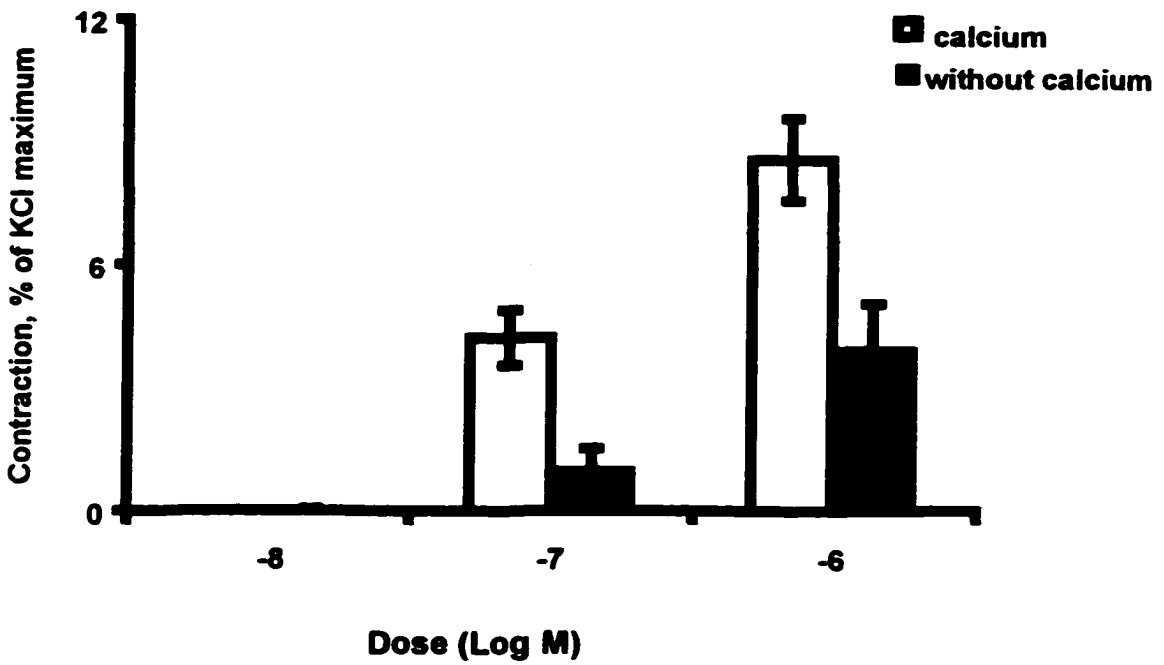


Fig-17: Effects of calcium-deficient buffer on DAMA-mediated contraction. The role of extracellular calcium was evaluated in response to DAMA doses (10^{-8}, 10^{-7}, 10^{-6} M). At low dose (10^{-8} M) DAMA did not demonstrate any measurable increase in contraction. However, at doses 10^{-7} & 10^{-6} M, the contractile response of DAMA in rings with extracellular calcium was greater as compared to the ones with no extracellular calcium, suggesting the involvement of both intracellular and extracellular calcium in DAMA-mediated contraction. This is a representative of n=4 experiments.

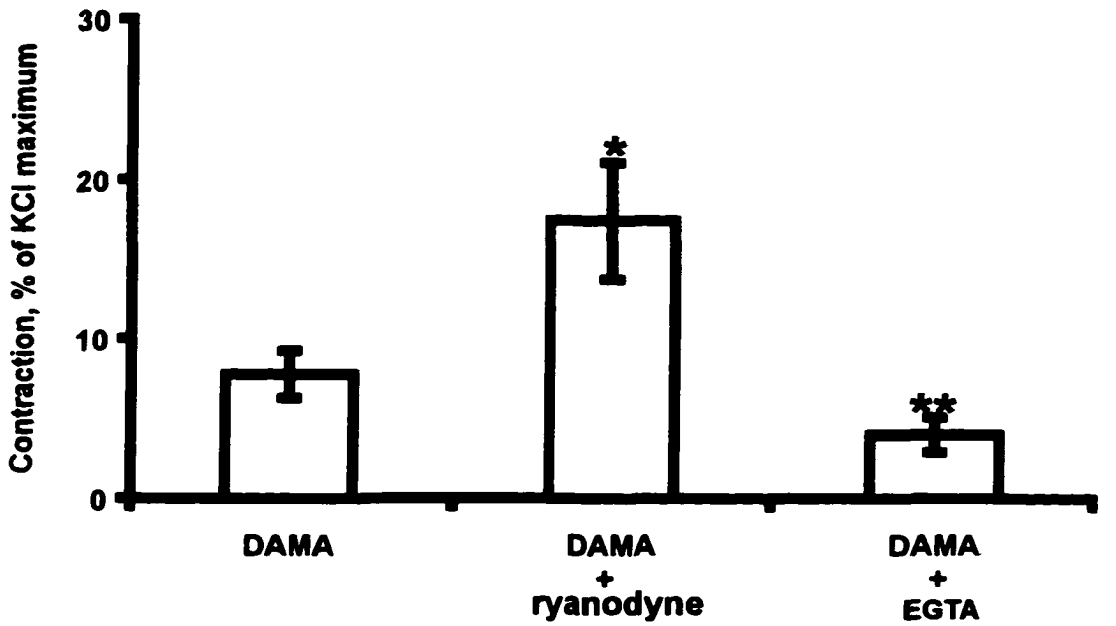


Fig-18: Effect of ryanodyne in agonist induced contraction in rat aorta. Pretreatment of 1μM ryanodyne caused an increase in DAMA-induced contraction suggesting the involvement of ryanodyne-sensitive stores of calcium in DAMA-mediated contractile response (*P<0.01, student's t-test). DAMA caused low level of contraction in calcium deficient buffer, suggesting that extracellular calcium may also contributed in DAMA-mediated contraction (see conclusion), (**P<0.05, Student's t-test). This is a representative of n=5 experiments.

CONCLUSION

We have demonstrated a functional delta opioid receptor in vascular smooth muscle. Our studies demonstrated that activation of the receptor induced cellular changes of physiological significance including: a) membrane depolarization; b) release of Ca^{2+} from intracellular stores and extracellular sources; c) tyrosine kinase activation; and finally d) aortic contraction. These data are not sufficient to identify the discreet steps in the signaling pathway between receptor activation and contraction. However, our findings coupled to existing data on DOR and smooth muscle function are sufficient to develop a working hypothesis and speculate about the complete signaling pathway that is utilized by DOR in vascular smooth muscle.

The physiological role of endogenous opioids on cardiovascular system has been well documented (Holaday JW, *et al.*, 1983; Champion HC, *et al.*, 1997; Parra L, *et al.*, 1995). The response may vary depending upon the species, anesthesia level and route of administration (Holaday JW, *et al.*, 1983). Furthermore, it has been documented that cross talk also occurs between mu and delta opioid receptors (Traynor JR, *et al.*, 1993). Furthermore a compensatory role of DAGO and DADLE inhibitory effect has been shown in the hypertensive rat to lower peripheral resistance which is increased during hypertension, suggesting a role of mu and delta opioid receptor in lowering hypertension (Wong SC, *et al.*, 1995). In contrast, our laboratory has failed to obtain evidence to suggest the presence of Mu or kappa opioid receptors in

vascular smooth muscle from arteries. Evaluation of A-10 cells by RT-PCR did not demonstrate transcripts for mu or kappa receptors. Furthermore mu or kappa agonists failed to induce contraction of aortic rings. Pre-incubation of the aortic rings with kappa receptor antagonists prior to stimulation with the DAMA failed to alter DAMA-mediated contraction, suggesting that other opioid receptors, if present, do not modulate DOR activity.

Endogenous opioid peptides are activated following stress and injury and modulate circulatory homeostatic mechanisms. The endogenous opioids appear capable of influencing circulatory responses to stress at the behavioral, endocrinological, and neural level. Our data presented in this dissertation suggests that opioid receptors are expressed on vascular smooth muscle in arteries and that in the aorta these receptors are functionally coupled to vascular contraction. Indeed, the cloned rat opioid receptors have been demonstrated to modulate ion channel conductivity and intracellular calcium levels, events that will have pronounced effects on vascular tone.

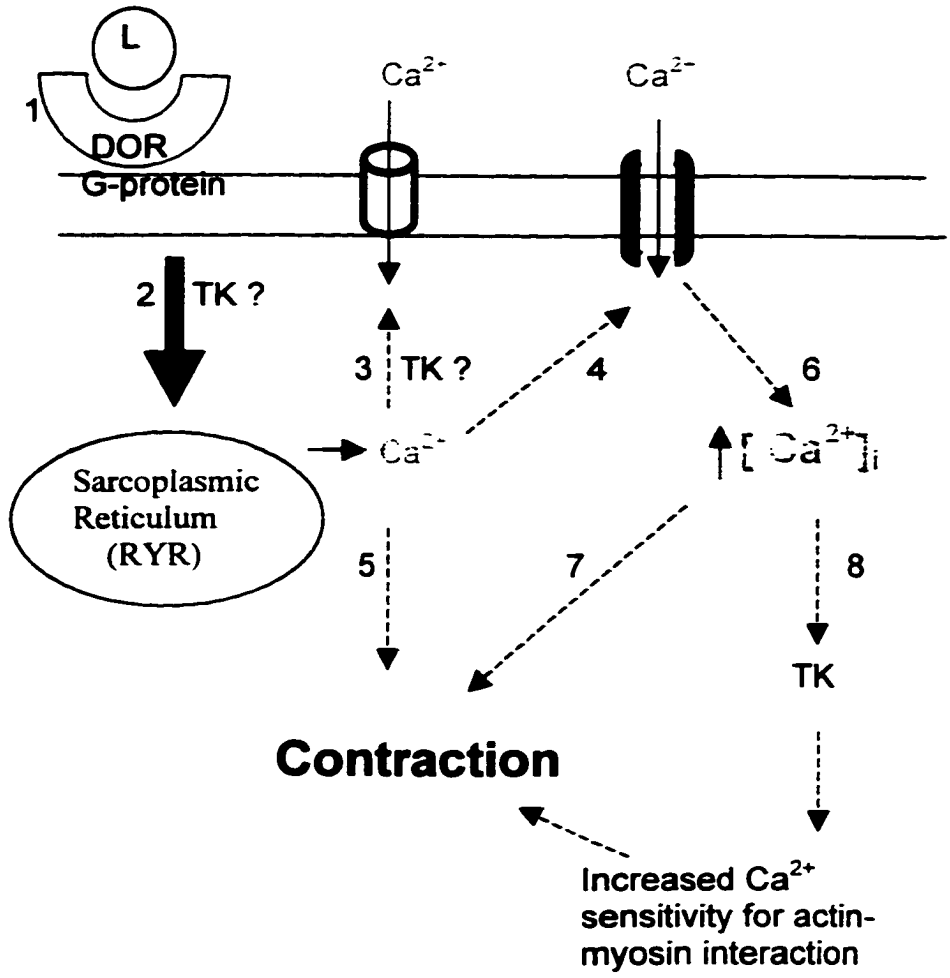


Fig-19: Proposed Mechanism of DOR activation in vascular smooth muscle: Representative scenario involving DOR activation and subsequent signaling mechanism. Ligand-receptor binding (1). Release of Ca^{2+} from RY-sensitive store (2). Release of Ca^{2+} from intracellular stores may cause an induction of Ca^{2+} from extracellular sources by tyrosine kinase (TK) pathway (3) and or by membrane depolarization (4) or it may directly cause contraction (5). The increase of Ca^{2+} from extracellular sources may potentiate contraction directly (7) and or by TK pathway, which increases the Ca^{2+} sensitivity for actin myosin interaction to cause contraction (8).

Our contraction studies suggest a slow developing vasoconstrictor effect that is primarily or exclusively dependent on DOR activation. Therefore, delta opioid receptor activation may potentiate the adverse effect of disease states where hypotension is observed. On the other hand it may provide the cardioprotective effect in disease conditions where hypertension is observed. Morphine is known to cause NO release from vascular endothelium by mu3-receptor (Stefano GB, *et al.*, 1995). This NO may diffuse across and cause relaxation of underlying smooth muscle. However, smooth muscle DOR activation could minimize or reverse the vasodilator effect of mu3-coupled NO release. Furthermore, opioid receptor activation has been demonstrated to block subsequent cNOS activation suggesting that DOR might promote changes in vascular tone by direct as well as indirect pathways. Finally, DOR may provide a compensatory mechanism to regulate the vascular tone at this site. Accomplishment of the signaling mechanism defines the normal functioning of vascular smooth muscle delta-opioid peptide receptor so that role of this receptor in various disease-states can be evaluated. These data, coupled to the previous reports that demonstrated increased circulating opioid levels following stress (Tanaka K, *et al.*, 1998; Fimiani C, *et al.*, 1999), and regulation of vascular response to hypoxia by delta opioids underscores the potential for DOR modulation of vascular smooth muscle function and may be of crucial importance in the treatment of cardiovascular dysfunction.

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